DETERMINATION OF PHENOLICS CONCENTRATION USING CROSS-LINKED PHENOL OXIDASE AGGREGATES

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

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The main object of the presented study was investigation of the use of cross-linked enzyme (tyrosinase) aggregates (CLTA) obtained from crude mushroom extract for a rapid phenolic content analysis in wines. In addition, a comparison of phenolic characteristics of Turkish red wines was performed. Reproducible and reliable results in total phenolic measurement were obtained with CLTAs similar to pure tyrosinase and tyrosinase obtained from crude mushroom extract. Measurement of total phenolic content is possible both in standard solutions and in complex matrices, such as wine. In a very short time period, 10 seconds, phenolics content in red and white wines produced from grapes of Turkey were investigated by using CLTAs. Results were consistent when compared to a well known phenolic measurement method, Folin-Ciocalteau. CLTAs exhibited very high operational stability and retained more than 90% of its activity after 30^{th} use. Moreover, it showed good shelf-life stability for about 2 months storage by maintaining 90% of its maximum activity. So, use of CLTAs prepared from crude mushroom extract is an effective, fast and cheap

alternative in total phenolics measurements in wines. Moreover, a novel catalase phenoloxidase (CATPO) produced by a fungal microorganism, *Scytalidium thermophilum*, was studied to check its capabilities in phenolics measurements. This novel catalase phenol oxidase showed similarly good results, exhibiting wide-substrate selectivity.

Keywords: Wine, phenol oxidase, immobilization, cross-linked tyrosinase aggregate (CLTA), phenolic compounds.

ÖZ

ÇAPRAZ BAĞLANMIŞ FENOL OKSİDAZ ÇÖKELTİLERİYLE FENOLİK KONSANTRASYON ÖLÇÜMÜ

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Bu çalışmanın amacı, ham mantar özütünden elde edilmiş çapraz bağlı enzim (tirosinaz) çökeltilerinin (ÇBTÇ) şaraplardaki hızlı fenolik madde tayininde kullanılımının araştırılmasıdır. Buna ek olarak, kırmızı Türk şaraplarının fenolik karakteristiklerinin karşılatırılması gerçekleştirilmiştir. ÇBTÇ'ler, saf tirosinaz ve ham mantar özütünden elde edilen tirosinazlarla benzer olarak toplam fenol miktarı ölçümünde güvenilir ve tekrarlanabilir sonuçlar vermiştir. Toplam fenol miktarının ölçümü hem standart solüsyonlarda hem de gerçek şarap örneklerinde mümkündür. Yöntemin toplam fenol miktarı ölçümünde 10 saniye gibi kısa bir reaksiyon süresi içinde gerçekleştirilmesi halinde dahi etkin olduğu kanıtlanmıştır. Çeşitli Türk üzümlerinden üretilen kırmızı ve beyaz şaraplardaki toplam fenol miktarları, 10 saniye gibi kısa bir sürede ÇBTÇ'ler kullanılarak ölçülmüştür. Sonuçların, iyi bilinen bir fenolik madde ölçüm yöntemi olan Folin-Ciocalteau metodu ile karşılaştırıldığında, tutarlı olduğu görülmüştür. ÇBTÇ'ler oldukça yüksek işlemsel kararlılık göstermiş ve 30. kullanımdan sonra bile aktivitesinin %90'ını korumuştur.

Ayrıca, 2 aylık bir sürede maksimum aktivitesinin %90'ınından fazlasını koruyarak, oldukça iyi bir raf ömrü kararlılığı göstermiştir. Şaraplardaki fenolik madde ölçümünde ÇBTÇ kullanımı etkili, hızlı ve düşük maliyetli bir alternatif yöntemdir. Buna ilaveten, *Scytalidium thermophilum* adlı fungal bir mikrooragnizma tarafından üretilen katalaz fenoloksidaz (KATFO), fenolik ölçümlerdeki kabiliyetleri açısından araştırıldı. Bu yeni katalaz-fenol oksidaz da düşük seçicilik sergileyerek benzer şekilde iyi sonuçlar ortaya koymuştur.

Keywords: Şarap, fenol oksidaz, tutuklama, çapraz bağlanmış tirosinaz çökeltileri (ÇBTÇ), fenolik bileşikler.

To My Parents

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I want express my deepest gratitude and love to my parents Nazire-Mustafa Ertürk for their encouragement, support and understanding all through the study.

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LIST OF ABBREVIATIONS

Abs	: Absorbance
Act	: Activity
APA	: Aminopenicillanic acid
AS	: Ammonium sulfate
CALB	: *Candida antarctica* Lipase B
CAT	: Catalase
CATPO	: Catalase-phenol oxidase
CCO	: Catalase-catechol oxidase
CLE	: Cross-linked enzymes
CLEA	: Cross-linked enzyme aggregates
CLEC	: Cross-linked enzyme crystals
CLTA	: Cross-linked tyrosinase aggregates
CSDE	: Cross-linked spray-dried enzymes
E	: Enzyme
GA	: Glutaraldehyde
HbHNL	: *Hevea brasiliensis* Hydroxynitrile Lyase
HNL	: Hydroxynitrile lyases
H_2O_2	: Hydrogen Peroxide
MBTH	: 3-methyl-2-benzothiazolinone hydrazone
MeHNL	: *Manihot Esculenta* Hydroxynitrile Lyase
O_2	: Oxygen
PAC	: Penicillin Acylase
PaHnL	: *Prunus amygdalus* Hydroxynitrile Lyase
PGA	: Phenylglycine amide

PO	: Phenol oxidase
PVPP	: Polyvinylpolypyrollidone
ROS	: Reactive oxygen species
U	: Enzyme activity unit
TPC	: Total Phenolic Content
YpSs	: Yeast protein soluble starch
ε	: Extinction coefficient

CHAPTER 1

INTRODUCTION

1.1 Wine Phenolics

Wine is a complex food product composed of 80–90% water and 8–14% ethanol. The remainder is comprised of hundreds of other components. Phenolics make up only 1-5% of the compounds found in wine, but are important due to their contribution towards appearance, taste, mouthfeel, and potential health benefits. The phenolic content of wine is influenced by many factors, including grape cultivar, environment, vineyard management and vinification practices (Lee and Tarara, 2007).

Importance and popularity of phenolic content of wines are due to two reasons: quality and health. Phenolic compounds are responsible for some major properties of wines, in particular, color and astringency. Thus optimization of phenolic contents of wine is important in terms of the quality of wine. Moreover, growing evidence of the role of free radicals and antioxidants in health and aging has focused great interest on these compounds. Free radicals are extremely harmful to living organisms in that they attack different constituents of the cell, which leads to acceleration of the aging process and sometimes, even cell destruction. Nutritional importance of polyphenols in wine is attributed to their activity, based on their ideal chemical structure for free radical scavenging (De Gaulejac *et al.,* 1999). In addition to that, many studies reported the inhibition of oxidation low density lipoproteins (LDLs) by phenolic compounds in red wine in vitro, demonstrating also that the consumption of beverages rich in polyphenols is associated to the increase in plasma antioxidant

potential and therefore to the prevention of coronary heart diseases (Frankel *et al.*, 1993;Kondo *et al.*, 1994)

Phenolics are secondary metabolites of plants, meaning they are not directly involved in plant's survival. Phenolics are formed from the essential amino acid, phenylalanine, thru the phenylpropanoid biosynthetic pathway. Phenolic compounds constitute a very diverse group; more than 4,000 have been identified. These compounds have been linked to several functions in plants: protection from UV radiation, pigmentation (coloration), defense against invading pathogens (antifungal properties), nodule protection, and attraction of pollinators and seed dispersers.

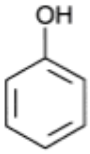
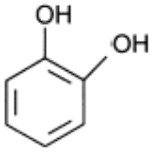
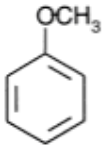
Phenols or phenolics include all the substances. *Simple phenols* (Table1.1) include those compounds that have a single aromatic ring containing one or more hydroxyl groups, a common example being caffeic acid. *Polyphenolic* compounds are those that have multiple phenol rings within the structure, and examples include catechin and ellagic acid. *Flavonoids* have a very specific three-ring structure. Tannin is a functional term which describes substances that are used to tan hide to leather. Many phenolic compounds have this property, although traditional phenol- containing tannins are natural plant extracts which contain a complex mixture of high molecular weight polyphenolic compounds. *Tannin* is often used loosely to describe high molecular weight phenolic mixtures. The term *condensed tannin* refers to mixtures of polymers of flavonoids and *hydrolyzable tannin* refers to gallic- or ellagic-acid–based mixtures, also called gallotannins or ellagitannins, respectively (Waterhouse, 2002).

The total amount of phenols found in a glass of red wine is on the order of 200 mg versus about 40 mg in a glass of white wine. Wine phenolics are grouped into two categories, the flavonoids and non-flavonoids.

1.1.1 Non-Flavonoids

In summary the non-flavonoids are listed with examples and general comments. Non-flavonoids, as the name would indicate, lack the flavan ring. These antioxidants found in wines have recently been of particular interest because they appear to help prevent arteries from becoming clogged with fatty blockages. Resveratrol is the non-flavonoid that researchers are most interested in.

Table 1.1 Simple Phenols

Structure	Names	Notes
	Phenol (hydroxybenzene)	Rare in natural products but abundant in sore throat sprays
	Pyrocatechol, (1,2 dihydroxybenzene). Note: when a 1,2 dihydroxy functional group is present, it is called a catechol group	Easily oxidized to *o*-quinone. Also called catechol: this same term has been used for catechin
	Anisole (methoxybenzene)	

1.1.1.1 Hydroxycinnamic Acids

These are the major phenols in grape juice and the major class of phenolics in white wine. These materials are also the first to be oxidized and subsequently initiate browning, a problem in white wines. There are three common hydroxycinnamates in grapes and wine, those based on coumaric acid (mono 4-hydroxy), caffeic acid

(catechol substitution), and ferulic acid (guaiacyl substitution) (Figure 1.1). Levels of total hydroxycinnamates in finished wine are typically 130 mg/L in whites and 60 mg/L in reds (Waterhouse, 2002).

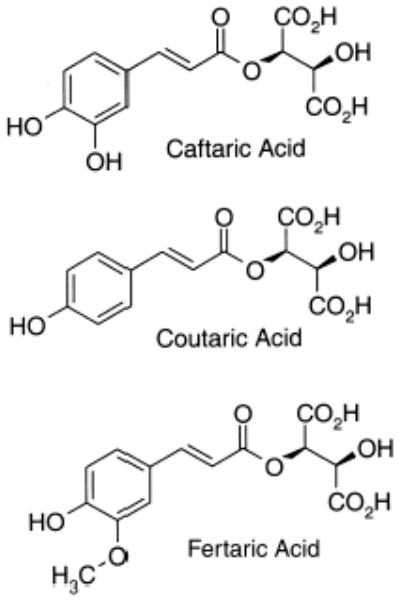


Figure 1.1 Three of the hydroxycinnamates in wine

1.1.1.2 Benzoic Acids

These are a minor component in new wines. Gallic acid appears from the hydrolysis of gallate esters of hydrolyzable tannins and condensed tannin after standing for at least a few months. Gallic acid appears to be stable during aging, as it is one phenolic compound readily visible by chromatographic analysis of older red wines. Its levels in red wine average near around 70 mg/L, while white wines average near 10 mg/L (Waterhouse and Teissedre, 1997). Gallic Acid is a powerful antioxidant, working to

stop free radical damage to cells. Tests showed that it inhibited cell proliferation and apoptosis (cell death) in prostate cancer cells (Waterhouse, 2002).

1.1.1.3 Hydrolyzable Tannins

Hydrolyzable tannins are ester-linked oligomers of gallic acid or ellagic acid with glucose or other sugars (Figure 1.2).

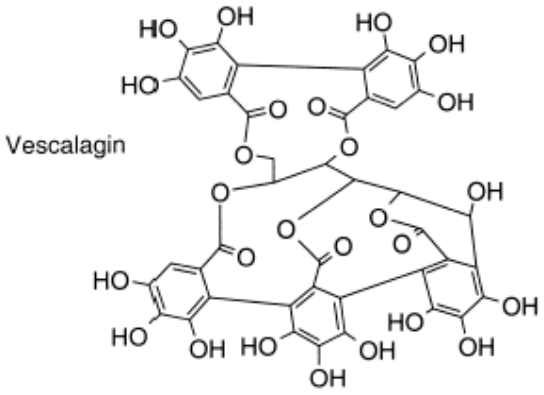


Figure 1. 2 An ellagitannin from oak

Because of the ester linkage, they are referred to as "hydrolyzable". There are two categories, the gallotannins and the ellagitannins which contain either gallic acid or ellagic acid (Waterhouse, 2002). Gallic acid is quite soluble and is found in all aged red wines, and it comes from both hydrolyzable tannins and the condensed tannins found in grape seeds. In wine hydrolyzable tannins come from oak and levels are near 100 mg/L for white wines aged for about 6 months in barrel, while red wines will have levels in the range of 250 mg/L after aging two or more years (Quinn&Singleton, 1985).

1.1.1.4 Stilbenes

Stilbenes are another minor class. Hydroxylated stilbenes found as the glycosides in grapes and a few other dietary sources (Waterhouse, 2002). Resveratrols attracted specific interest because of their potential therapeutic biological attributes that favour protection against atherosclerosis, including antioxidant activity, modulation of hepatic apolipoprotein and lipid synthesis as well as inhibition of platelet aggregation (Balsy *et al.*, 2000). Several forms of resveratrol exist including the *cis* and *trans* isomers as well as the glucosides of both isomers (Figure.1.3).

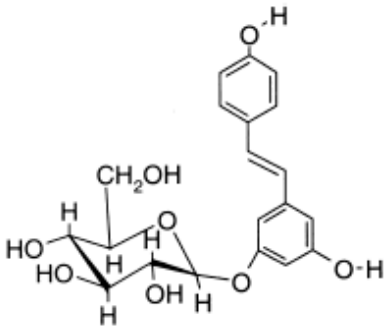


Figure 1. 3 *Trans*-piceid, the resveratrol glucoside

Resveratrol derivatives are found only in the skin of the grape, so much more is found in red wine. The total levels of all forms average about 7 mg/L for reds, (Lamuela-Raventós *et al*, 1995), 2 mg/L for rosés and 0.5 mg/L for white wines (Romero-Perez *et al*, 1996).

1.1.2 Flavonoids

Flavonoids get their name from the fact that they share a unit in their skeleton called a flavan ring. The flavonoids comprise the majority of the phenols in red wine and are derived from extraction of the skins and seeds of grapes during the fermentation process. Since red wines are produced by fermentation of the juice's sugar into alcohol—a good solvent for polyphenol extraction—in the presence of the skins and seeds over a period of 4–10 or more days, there is ample opportunity for extraction of much of the polyphenols into red wine, and in typical wine making, about half these substance are extracted during the maceration process. The major classes of wine flavonoids are the flavanols, flavonols, and the anthocyanins (Waterhouse, 2002).

1.1.2.1 Flavanols

Flavonols are the most abundant class of flavonoids in grapes and wine, and in the grape they are found in both the seed and skin. These are often specifically called the flavan-3-ols to identify the location of the alcohol group on the 3rd ring. The *Trans* form is (+)-catechin and the *Cis* form is (-)-epicatechin (Figure 1.4). Epigallocatechin is found in grape skin. Epicatechin gallate is a small but significant proportion of the flavan-3-ol pool in grapes in the seeds. Thus, for the simple series of monomeric flavanols, there are four different ones found in wine. These monomeric flavan-3-ols are sometimes referred to as the "catechins." The levels of total monomeric flavan-3-ols in typical red wine are in the range of 40–120 mg/L with the majority usually being catechin (Ritchey *et al*., 1999).

According to the Robichaud *et al.* (1990), the monomeric catechins are bitter and astringent. However, in the polymer, the bitterness is minimal, but the astringency remains.

The levels are strongly affected by seed extraction techniques and are higher when extended maceration techniques are used. The majority of phenolic compounds in red wine are from the condensation of flavan-3-ol units to yield the oligomers (proanthocyanidins) and polymers (condensed tannins).

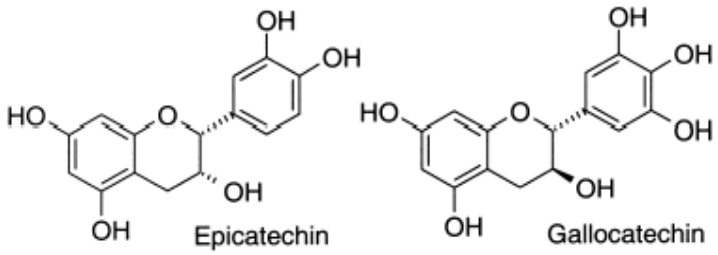


Figure 1. 4 *Cis* and *trans* forms of flavan-3-ols.

On average, epicatechin is the predominant unit in condensed tannins from grapes and wine, catechin is the next most abundant. In typical red wines, the amount of polymer plus oligomer is a sizable fraction of the total phenolic level, being in the range of 25 to 50% in new wines and a higher proportion in older wines. Levels are between 0.5 g/L and 1.5 g/L or even higher in some red wines, while in white wine, levels are in the range of 10–50 mg/L and highly dependent on pressing techniques. These substances are unlikely to have many health effects, except in the gut, as they appear to not be absorbed (Donovan *et al.*, 2002) owing to their high molecular weight (Schramm *et al.*, 1998).

1.1.2.2 Flavonols

There are only three forms of the simple flavonoid aglycones in grapes, quercetin (see Figure 1.5), myricetin (3'4'5'trihydroxy) and kaempferol (4′hydroxy), but since these compounds occur with a diverse combination of glycosidic forms, there are many individual compounds present. Cheynier *et al.* (1986) claimed that, they have been shown to be mostly the 3-glucosides as well as the 3-glucuronides and small amounts of diglycosides in Cinsault.

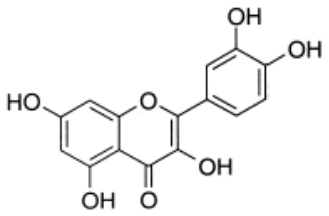


Figure 1. 5 Quercetin

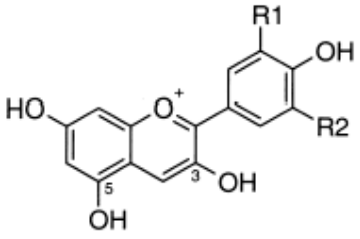
A study on Pinot noir by Price *et al*. (1995) has shown that sunlight on the berry skin strongly enhances the levels of the flavonols. Although this has not been studied in other grape varieties, a study of the levels of phenolic compounds in wine has shown that expensive Cabernet Sauvignon wines expected to come from lower yielding vines with better sun exposure of the fruit has at least three-fold higher levels of flavonols, suggesting that the levels of these compounds in grapes may be a useful indicator of grape sun exposure and perhaps quality. Levels of total flavonols are near 53 mg/L for widely sold Cabernet wine, but over 200 mg/L in more expensive wine (Ritchey *et al*., 1999).

1.1.2.3 Anthocyanins

Anthocyanins provide the color in red wine and the red and blue colors found in the skins of red or black grapes, but also in many other plants including other foods in the diet. The anthocyanins react with the tannins to produce a "stabilized" anthocyanin or pigmented tannin which persists much longer in wine than the initial form, and it is this stabilized color that persists in most red wines more than a few years old, although in some wines the monomeric forms found in the grape are still found after this time.

The term for the simple flavonoid ring system is anthocyanidin. There are five basic anthocyanidins in wine: cyanidin, peonidin, delphindin, petunidin and malvidin, the most abundant in red wines (Figure 1.6) "Anthocyanin" implies a glycoside (Figure 1.7) (Waterhouse, 2002).

In wine and similar solutions there are several forms of anthocyanins and the proportions strongly affect the color of a solution containing these substances (Figure 1.7). There is a quinone form which has a violet hue, and its pKa is 4.7, so it is present in small amounts at high wine pH values.



R1, R2
H, OH; cyanidin
H, OCH_3; peonidin
OH, OH; delphinidin
OH, OCH_3; petunidin
OCH_3, OCH_3; malvidin

Figure 1. 6 Anthocyanidin Structures

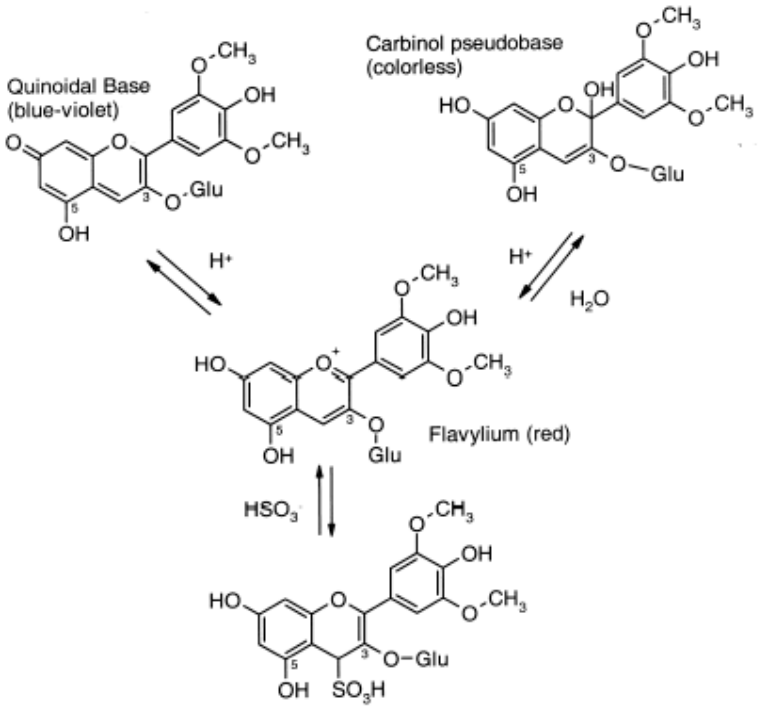


Figure 1. 7 Anthocyanin forms present in wine.

1.1.3 Phenolic Levels in Wines

Different grape species will have some different phenolic compounds as well as different phenolic levels. The level of the phenolics in wines is also highly variable due to both differences in fruit sources as well as processing. The first and most important factor to understand is that white wines are made by quickly pressing the juice away from the grape solids, while reds are made by fermenting the juice in the presence of the grape solids (skin and seeds). Since the skins and seeds contain the most of the phenols, red wine is a whole berry extract while white wine is a juice product. In addition, because of the significant sensory effects of these substances on

bitterness and astringency, they are controlled by wine makers. Although there is a wide range of variation, typical levels for red and white wines are listed in Table 1.2 based on literature sources noted in the sections above (Waterhouse, 2002).

1.2 Detection and quantification of phenolic compounds

The modern wine-making industry is focused on controlling total polyphenol (TPC) as polyphenols are responsible for the sensory properties of wine. Increasing interest on polyphenols is providing an impetus for the development of analytical techniques for fast monitoring of these compounds. Traditionally, analysis has been based on spectrophotometric or chromatographic methods. New techniques that are currently being developed include capillary electrophoresis, immunoassays, and biosensors. They potentially provide better specificity, lower costs, and faster and simpler sample processing (Yamada *et al., 2005*).

Biosensors are currently being developed for the detection of phenolic compounds, based on the reaction of these compounds with an immobilized mushroom tyrosinase. Immobilization is essential because it ensures intimate contact between the enzyme and the underlying signal detector and also prevents the enzyme from being washed off the electrode when readings are made in aqueous samples (Tembe *et al*., 2006).

The detection and quantification of the reaction within the biosensor can be based on various different principles, such as detection of oxygen consumption (Campanella *et al.*, 1992), direct increment of liberated *o*-quinone (Hall *et al*., 1988; Wang *et al*., 1994; Lutz *et al*., 1995; Önnerfjord *et al.,* 1995; Li *et al*., 1998) or increment of the o-quinone using a redox mediator such as hexacyanoferrate (Bonakdar *et al.,* 1989).

Table 1. 2 Typical levels of phenolics in red and white table wine[a] (Waterhouse, 2002)

	White Wine		Red Wine	
Phenol Class	Young	Aged	Young	Aged
Non-flavonoids				
Hydroxycinnamates	154	130	165	60
Benzoic Acids	10	15	60	60
Hydrolyzable tannins (from oak)	0	100	0	250
Stilbenes (Resveratrol)	0.5	0.5	7	7
Total mg/L	**164.5**	**245.5**	**232**	**377**
Flavonoids				
Flavanol monomers	25	15	200	100
Proanthocyanidins and condensed tannins	20	25	750	1,000
Flavonols	—	—	100	100
Anthocyanins	—	—	400	90
Others	—	—	50	75
Total mg/L	**45**	**40**	**1,500**	**1,365**
Total all phenols	**209.5**	**285.5**	**1,732**	**1,742**

[a] Young means new wine, less than six months of age, not having been aged or fermented in oak barrels. Aged implies about one year for white, about two years for red and some oak barrel aging (or other oak contact).

1.3 Phenoloxidases

Phenoloxidases (PO) are oxidoreductases that catalyze oxidation of phenolic compounds (Durán and Esposito, 1997). In any enzyme assay, a specified amount of PO can be used for quantitative oxidation of TPC. They are subdivided into two subclasses, laccases and tyrosinases, and both groups react with oxygen and no cofactors are needed (Steffens *et al.*, 1998; Chevalier *et al.*, 1999).

1.3.1 Laccase

Laccases (EC 1.10.3.2, *p*-diphenol:dioxigen oxidoreductase) was first described by Yoshida (1883) and was characterised as a metal containing oxidase by Bertrand (1985). They are cuproproteins that catalyse the oxidation of several aromatic and inorganic substances (phenols) with the concomitant reduction of oxygen to water (Xu, 1996). Laccases are characterised by low substrate specificity and their catalytic competence varies widely depending on the source.

1.3.2 Tyrosinase

Tyrosinases are type-3 copper proteins involved in the initial step of melanin synthesis. Tyrosinases are involved in the melanin pathway and are especially responsible for the first steps of melanin synthesis from L-tyrosine leading to the formation of L-dopaquinone and L-dopachrome (Sanchez-Ferrer *et al.*, 1995). The particularity of tyrosinases is to catalyse the o-hydroxylation of monophenols (cresolase activity or ‘‘monophenolase’’) and the subsequent oxidation of the resulting o-diphenols into reactive o-quinones (catecholase activity or ‘‘diphenolase’’), both reactions using molecular oxygen (Figure 1.8). Subsequently, the o-quinones undergo non-enzymatic reactions with various nucleophiles, producing intermediates, which associate spontaneously in dark brown pigments (Soler-Rivas *et al.* 1999).

In fungi, tyrosinases are generally associated with the formation and stability of spores, in defence and virulence mechanisms, and in browning and pigmentation. Mushroom tyrosinase is popular among researchers as it is commercially available and inexpensive and also there are easy tools to investigate the feature of this enzyme. First characterized from the edible mushroom *Agaricus bisporus* (Nakamura *et al.* 1966, Robb and Gutteridge 1981, Wichers *et al*. 1996) because of

undesirable enzymatic browning problems during post harvest storage (Jolivet *et al.* 1998), tyrosinases were found, more recently, in several other fungi with relevant insights into molecular and genetic characteristics and intoreaction mechanisms, highlighting their very promising properties for biotechnological applications (Halaouli *et al.* 2006). Among mushrooms *A. bisporus* is the most commonly consumed species worldwide, and also it is a representative of its family; for this reason most of the research work is being carried out on this particular species.

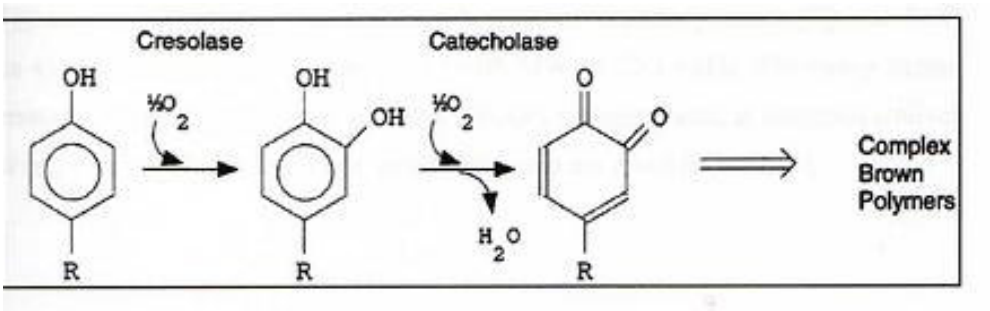


Figure 1. 8 Reaction mechanism of tyrosinase with phenol in the presence of oxygen

Laccase shows only low substrate specificity, whereas tyrosinase catalyzes two reactions: first, the oxidation of monophenols to o-diphenols and second, the oxidation of o-diphenols to o-quinones. Because of these properties, tyrosinase was chosen as the biological component for further studies. The described activities of tyrosinase ensure that all contained polyphenols can be measured while side reactions can be avoided (Seo *et al*., 2003; Fenoll *et al.* 2001;Duran *et al.,* 2002) Moreover, all of the tyrosinases obtained from various species of mushroom have similar properties; analyzed tyrosinases of this study are related to *A. bisporus*. Mushroom (*Agaricus bisporus*) tyrosinase is popular among researchers because it is commercially available, inexpensive and there are easy tools to investigate the feature of this enzyme.

1.4 Catalases

Catalases (EC 1.11.1.6) are a group of metalloenzymes with the major function for degradation of hydrogen peroxide (H_2O_2) to dioxygen and water (Goldberg and Hochman, 1989). This function is essential to control hydrogen peroxide levels in living systems, where hydrogen peroxide is a by-product of mitochondrial electron transport, β-oxidation of fatty acids, and photorespiration (Montavon *et al*. 2007). Fungi are effective producers of these enzymes (Isobe *et al*. 2006) possessing catalases, peroxidases, and superoxide dismutases to ensure that reactive oxygen species (ROS) like H_2O_2, superoxide, hydroxyl radicals, and singlet oxygen are maintained at safe concentrations (Calera *et al*. 2000).

1.5 Bifunctionality of Catalases and Phenol Oxidases

As explained previously, the major function of catalases is the degradation of hydrogen peroxide to dioxygen and water. Secondary function of catalases is reported as the oxidation of hydrogen donors such as methanol, ethanol, formic acid, phenols, with the consumption of peroxide, called peroxidic activity (Packer, 1984). The third and novel function of catalases; oxidase activity in the absence of hydrogen peroxide, was introduced by Vetrano *et al.* (2005) for mammalian catalases.

A new group of catalases, the catalase-phenol oxidases (CATPO), was introduced by Sutay-Kocabaş *et al*.(2008). According to Sutay-Kocabaş *et al.* CATPOs are capable of hydrogen peroxide decomposition (catalase activity) and phenolic (o-diphenolic compound, especially catechol) oxidation in the absence of hydrogen peroxide (phenol oxidase activity).

Here, we investigate the use of a bifunctional enzyme, catalase-phenol oxidase (CATPO) from the thermophilic fungus, *S. thermophilum*. Previously, this enzyme

was reported as an extracellular phenol oxidase (Ogel *et al.* 2006). However, after purification, characterization, and amino acid sequencing, the enzyme was revealed to be a catalase, displaying an additional phenol oxidase activity by Sutay-Kocabaş *et al*. (2008).

1.6 Immobilization

Enzymes have a number of distinct advantages over conventional chemical catalysts. Foremost amongst these are their specificity and selectivity not only for particular reactions but also in their discrimination between similar parts of molecules (regiospecificity) or optical isomers (stereospecificity). However, there are some disadvantages in the use of enzymes which cannot be ignored but which are currently being addressed and overcome. In particular, the high cost of enzyme isolation and purification still discourages their use, especially in areas which currently have an established alternative procedure. The generally unstable nature of enzymes, when removed from their natural environment, is also a major drawback to their more extensive use. These drawbacks can be eliminated by enzyme immobilization (Chaplin and Bucke, 1990).

Immobilization is the conversion of enzymes from a water-soluble, mobile state to a water-insoluble, immobile state. It prevents enzyme diffusion in the reaction mixtures and facilitates their recovery from the product stream by solid liquid separation techniques. The advantages of immobilization are (1) multiple and repetitive use of a single batch of enzymes; (2) creation of buffer by the support against changes in pH, temperature and ionic strength in the bulk solvent, as well as protection from shear forces; (3) no contamination of processed solution with enzyme; and (4) analytical considerations, especially with respect to long-life for activity and predictable decay rates. Mass transfer limitations are usually observed in immobilized enzymes. In some instances, these limitations can be advantageous, for example, when substrate inhibition takes place (Birnhaum, 1988). As results of these

advantages, operational lifetime and stability enhances, and cost of the process reduces. Moreover, improved enzyme performance, such as activity, stability and selectivity, can often be achieved by enzyme immobilization. (Clark, 1994; Cabral *et al.,* 1993; Rocchietti *et al*., 2002)

Immobilized enzymes are divided into two groups; carrier-bond and carrier-free (Cao *et al*., 2003)

1.6.1 Carrier-bound Immobilization

Since the early days of enzyme immobilization, the primary goal of using insoluble carriers of ready-made geometric parameters and defined physical and chemical nature has been to insolubilise the enzyme and thus to facilitate its separation and use (Messing, 1975). The various methods used in this type of immobilization are: adsorption, covalent coupling, entrapment, and encapsulation (Birnhaum, 1988).

Adsorption is based on ionic or hydrophobic interactions between the protein and supporting carrier. Since the binding is reversible, the support is regenerable. Adsorption is simple technique but since in this method forces between enzyme and support are relatively weak, leakage of the enzyme from the support occurs (Birnhaum, 1988; Walsh, 2002). Covalent Coupling overcomes this, but harsh conditions lead to undesirable loss of activity (Walsh, 2002). Entrapment of proteins within polymer network is another method of carrier-bound immobilization. Entrapment can be applied to only low molecular weight substrates and products due to diffusion limitations. The other method is encapsulation, which is achieved by physical confinement of enzymes by using membranes (Birnhaum, 1988).

There are many disadvantages of using carrier-bound immobilization methods. First, the use of extra polymers as carrier leads to ‘dilution of activity’ of immobilized

enzymes, owing to the introduction of a large portion of non-catalytic mass ranging from 99.9% to 90%. This leads to lower space-time yields and lower productivity. In addition to that, the design of the carrier-bound immobilized enzymes requires laborious and time consuming trial and error experiments. Moreover, immobilization of an enzyme on a carrier often leads to the loss of more than 50% native activity, especially at high enzyme loadings. Last, carriers where the catalyst is immobilized are not accesible enough for a fast activity test (Cao *et al*., 2003).

1.6.2 Carrier-free Immobilization

There is no need for extra inactive mass as carrier in carrier-free type immobilization (Cao *et al.*, 2003). As a result, these immobilized enzymes have high activities (Wilson *et al.*, 2004). Generally prepared by directly cross-linking different enzyme preparations such as: dissolved enzyme, cyrstalline enzyme, spray dried enzyme and physically aggregated enzyme resulting in formation of CLEs, CLECs, cross-linked spray dried enzymes (CSDEs) (Amotz, 1987) and CLEAs, respectively (Cao *et al*., 2003) Thus, they are discriminated from each other only by the precursors used for cross-linking, as illustrated in Figure 1.9.

1.6.2.1 CLEs

CLEs are formed by cross-linking of dissolved enzyme with a bifunctional chemical cross-linker, such as glutaraldehyde (Quiocho *et al.*, 1966). Enhanced thermostability could be obtained by the cross-linking of dissolved enzyme. However, intermolecular cross-linking of these highly solvated enzyme molecules often had several drawbacks, such as lower activity retention (usually below 50% of the native activity), poor reproducibility and low mechanical stability. Moreover, handling of gelatinous CLE is another disadvantage. (Cao *et al*., 2003)

1.6.2.2 CLECs

Cross-linking of highly compacted enzyme crystals leads to the formation of insoluble CLECs with considerable activity (Quiocho *et al*., 1964). CLECs exhibited enhanced thermostability and mechanical stability relative to amorphous CLE (Quicho *et al.*, 1966). They also had broad pH stability (Tüchsen *et al*., 1977) and showed enhanced stability against organic solvents (Lee *et al*., 1986). However, CLECs have an inherent disadvantage: enzyme crystallization is a laborious procedure requiring enzyme of high purity, which translates to prohibitively high costs. Furthermore, this method has narrow applicability (Cao *et al*., 2003).

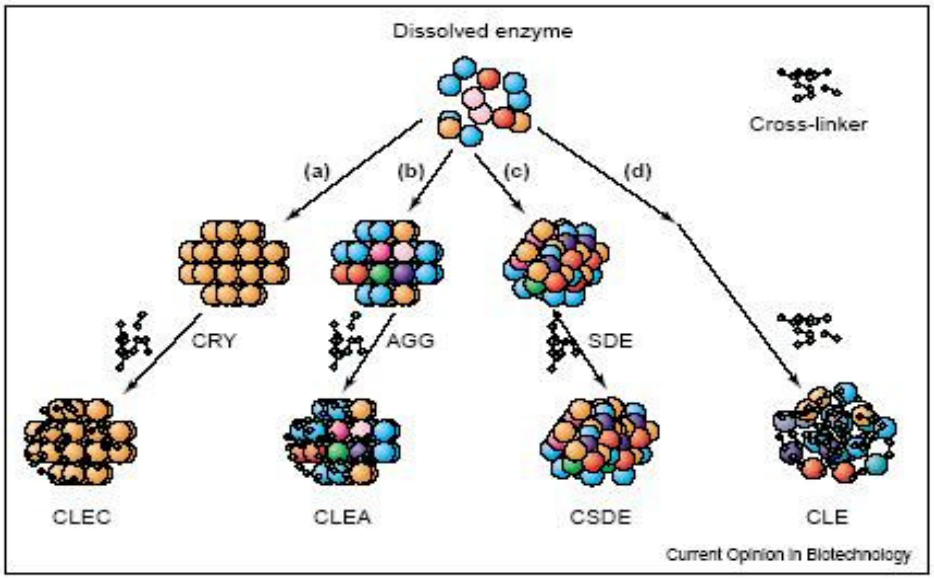


Figure 1. 9 The different approaches to the production of carrier-free immobilised enzymes: (a) crystallization; (b) aggregation; (c) spray-drying; (d) direct cross-linking. AGG, aggregates; CRY, crystals; SDE, spray-dried enzyme. (Cao *et al*., 2003)

1.6.2.3 CLSDs

Spray-dried enzyme powders were also used to prepare cross-linked enzymes (Amotz, 1987). Although reasonable activity was obtained, this approach has not been exploited until now, owing to the fact that the spray-drying process reversibly deactivates the enzymes. Consequently, compared with CLECs, or carrier-bound immobilized enzymes, CLSDs display relatively low activity (Cao *et al.*, 1999).

1.6.2.4 CLEAs

More recently, CLEAs were added to the family of carrier-free immobilized enzymes (Cao *et al*., 2000). In this method, enzymes were precipitated without disturbing their three-dimensional structure by changing pH or ionic strength by adding appropriate aggregation agents, such as those widely used in non-denaturing protein separation (Rothstein, 1994). These insoluble physical aggregates can be subsequently cross-linked by the addition of bifunctional cross-linkers (Cao *et al*., 2003).

The CLEAs formed exhibited comparable activities and stabilities to CLECs (Cao *et al*., 2000; Cao *et al*., 2001). As protein aggregation is the most frequently used primary method of protein purification, the preparation of CLEAs obviates the need for a highly purified enzyme and the labor-intensive crystallization step (Cao *et al*., 2003). Moreover, there is no need for laborious optimization of the crystal form (Margolin, 1996). There is also opportunity to co-immobilize different enzymes for use in one-pot, multi-step synthesis. In addition to that, it is a promising approach to render enzymes tolerant to denaturing ionic liquids. Other benefits of the method are low cost and fast optimization opportunities (Van Langen *et al.*, 2005).

1.6.3 Applications of CLEAs

The use of CLEAs might by beneficial for processes where high productivity and space-time yield are required or for use with labile enzymes that cannot be efficiently stabilized by means of conventional carrier-bound immobilization methods. Moreover, it is clearly advantageous in that it is possible to feed the reactor with more enzymes, to compansate for the loss of activity during recycling, without prolonging the reaction time. Their high stability renders them suitable for enzyme-catalyzed biotransformations and varying aggregation conditions, leading to an increase in enantioselectivity and activity.

CLEAs are very attractive biocatalysts, owing to their facile, inexpensive and effective production method. They can readily be reused and exhibit improved stability and performance. The methodology is applicable to essentially any enzyme, including cofactor dependent oxidoreductases (Sheldon *et al*., 2007). Application to penicillin acylase used in antibiotic synthesis showed large improvements over other type of biocatalysts (Illanes *et al*., 2006). The potential applications of CLEAs are numerous and include:

- Synthesis of pharmaceuticals, flavors and fragrances, agrochemicals, nutraceuticals, fine chemicals, bulk monomers and biofuels.
- Animal feed, *e.g.* phytase for utilization of organically bound phosphate by pigs and poultry.
- Food and beverage processing, *e.g.* lipases in cheese manufacture and laccase in wine clarification.
- Cosmetics, *e.g.* in skin care products
- Oils and fats processing, *e.g.* in biolubricants, bioemulsifiers, bioemollients.
- Carbohydrate processing, *e.g.* laccase in carbohydrate oxidations.
- Pulp and paper, *e.g.* in pulp bleaching.

- Detergents, *e.g.* proteases, amylases and lipases for removal of protein, carbohydrate and fat stains.
- Waste water treatment, *e.g.* for removal of phenols, dyes, and endocrine disrupters.
- Biosensors/diagnostics, *e.g.* glucose oxidase and cholesterol oxidase biosensors.
- Delivery of proteins as therapeutic agents or nutritional/digestive supplements *e.g.* beta-galactosidase for digestive hydrolysis of lactose in dairy products to alleviate the symptoms of lactose intolerance.

The studies based on preparation of cross-linked enzyme aggregates of several enzymes and their applications are demonstrated in Table 1.3.

Table 1. 3 CLEAs of Different Enzymes and Their Application Areas

Article	Year	Enzyme	Application	Conclusion
Cross-Linked Enzyme Aggregates: A Simple and Effective Method for the Immobilization of Penicillin Acylase **(Cao *et al.*)**	2000	Penicillin G Acylase	Synthesis of ampicillin From D-phenylglycine amide (PGA) and 6-aminopenicillanic acid (6-APA)	CLEAs of penicillin G acylase, were more efficient catalysts than cross-linked crystals (CLECs) of the same enzyme, with a high stability and activity
Cross-linked aggregates of penicillin acylase: robust catalysts for the synthesis of β-lactam antibiotics **(Cao *et al.*)**	2001	Penicillin G Acylase	Synthesis of ampicillin in aqueous media by CLECs, PGA-450, CLEAs	CLEAs of PGA combine the high volumetric activity and a high S/H ratio in β-lactam synthesis with a higher stability and activity
Penicillin Acylase Catalysed Synthesis of Ampicillin in Hydrophilic Organic Solvents **(van Langen *et al.*)**	2003	Penicillin acylase from *Alcaligenes faecalis*	Synthesis of ampicillin from D-PGA and 6-APA in the presence of an *A. faecalis* pencillin acylase CLEA	86% yield
Cross-Linked Aggregates of (*R*)-Oxynitrilase: A Stable, Recyclable Biocatalyst for Enantioselective Hydrocyanation **(van Langen *et al.*)**	2005	Hydroxynitrile Lyase Prunus amygdalus (PaHnL)	Hydrocyanation of 4 aldehydes (2-methylbenzaldehyde, 2-Chlorobenzaldehyde, 2-Nitrobenzaldehyde, trans-cinnamaldehyde)	PaHnL CLEA is a stable and recyclable biocatalyst and superior for enantioselective hydrocyanation

Table 1.3 (cont'd) CLEAs of Different Enzymes and Their Application Areas

Article	Year	Enzyme	Application	Conclusion
Galacto-oligosaccharide synthesis by immobilized Aspergillus oryzae β-galactosidase **(Gaur *et al.*)**	2006	Aspergillus oryzae β-galactosidase	Galacto-oligosaccharide synthesis by three different immobilization techniques	Cross-linked aggregation of β-galactosidase showed higher enzyme recovery and more stable than free enzyme
Synthesis of enantiomerically pure (S)-mandelic acid using an oxynitrilase–nitrilase bienzymatic cascade: a nitrilase surprisingly shows nitrile hydratase activity **(Mateo *et al.*)**	2006	(S)-selective oxynitrilase from M.esculenta and the non-selective recombinant nitrilase from P.fluorescens	Conversion benzaldehyde into enantiomerically pure (S)-mandelic acid in the presence of a CLEA	>94% conversion 95% ee
Cross-linked Penicillin Acylase Aggregates for Synthesis of β-Lactam Antibiotics in Organic Medium **(Illanes *et al.*)**	2006	Partially purified Escherichia Coli Penicillin Acylase	Synthesis of β-Lactam Antibiotics by CLEA and carrier bound PA biocatalysts	Penicillin Acylase CLEAs are very powerful biocatalysts for the synthesis of β-lactam antibiotics in organic media
Continuous kinetic resolution catalysed by cross-linked enzyme aggregates, ' CLEAs', in supercritical CO_2 **(Hobbs *et al.*)**	2006	*Candida antarctica* Lipase B (CALB)	To catalyse the kinetic resolution of tetralol and 1-phenylethanol in a continuous supercriticalCO_2	CALB-CLEA is a superior catalyst for this kinetic resolution in scCO_2 compared to native CALB and Novozym 435 in scCO_2

Table 1.3 (cont'd) CLEAs of Different Enzymes and Their Application Areas

Article	Year	Enzyme	Application	Conclusion
Immobilized hydroxynitrile lyases for enantioselective synthesis of cyanohydrins: sol-gels and cross-linked enzyme aggregates **(Cabirol *et al.*)**	2006	Hydroxynitrile Lyases (PaHNL, MeHNL, HbHNL)	Enantioselective synthesis of cyanohydrins with immobilized enzymes (CLEAs and Sol-gels)	MeHNL-CLEA proved to be a remarkably robust and efficient biocatalyst in diisopropyl ether.
Cross-linked enzyme aggregates (CLEAs) with controlled particles: Application to *Candida rugosa* lipase **(Yu *et al.*)**	2006	*Candida rugosa* lipase	-	The carrier-free immobilized *C. rugosa* lipase can keep lipase activity quite well and enantioselectivity of the lipase is able to be improved after immobilized by CLEA
Cross-Linked Aggregates of the Hydroxynitrile Lyase from Manihot esculenta: Highly Active and Robust Biocatalysts **(Chmura *et al.*)**	2006	*Hydroxynitrilase from M. Esculenta (Me-HNL)*	Hydrocyanation of aldehydes and ketones by MeHNL-CLEA	>90% conversion

Table 1.3 (cont'd) CLEAs of Different Enzymes and Their Application Areas

Article	Year	Enzyme	Application	Conclusion
Production of cephalexin in organic medium at high substrate concentrations with CLEA of penicillin acylase and PGA-450 **(Illanes *et al.*)**	2007	Penicillin Acylase	Production of cephalexin with CLEA of penicillin acylase	40.1g and 135.5g of cephalexin/g of biocatalyst were obtained for PGA-450 and CLEA, respectively
Asymmetric synthesis of cyanohydrin derived from pyridine aldehyde with cross - linked aggregates of hydroxynitrile lyases **(Roberger *et al.*)**	2007	Two different Hydroxynitrile Lyases (MeHNL-PaHNL)	Production of cyanohydrins from 3-pyridinecarboxaldehyde by HNL-CLEAs	>65% yield >93% ee
Application of cross - linked enzyme aggregates of *Bacillus badius* penicillin G acylase for the production of 6-aminopenicillanic acid **(Rajendhran *et al.*)**	2007	Penicillin G Acylase (PAC)	Conversion of penicillin G to 6-APA by CLEA-PAC	80% immobilization yield Conversion in 60 min Reusable for 20 times with 100% activity
Preparation of cross-linked tyrosinase aggregates **(Aytar *et al.*)**	2008	Tyrosinase	-	100% enzyme recovery

1.6.4 CLEAs of Tyrosinase

Aytar *et al.* (2008) prepared and characterized the CLEAs of tyrosinase from crude extracts of mushroom (*Agaricus bisporus*) for the first time. In this study, mushroom tyrosinase was immobilized in a simple and effective way by physical aggregation of the enzyme, using ammonium sulfate as precipitant, followed by chemical cross-linking by glutaraldehyde to form insoluble cross-linked enzyme aggregates (CLEAs). In the optimization studies, the effects of protein addition, AS and GA addition, and cross-linking temperature and period on the enzyme recovery of CLEAs were investigated. Optimum conditions for production of CLEAs of tyrosinase from crude mushroom extract were shown in Table 1.4.

CLEAs prepared from crude mushrrom tyrosinase under the optimum conditions, had nearly 100% enzyme recovery. According to Aytar *et al*. (2008), CLEAs of crude tyrosinases still retained 72% of their maximum activity after an 8 month of storage at 4°C, while crude mushroom tyrosinase retained 2% of its activity after 3 months of storage at 4°C.

In the characterization studies, she found that the optimum temperature and activation energy of catechol oxidation for both the CLEA and free enzyme were 34°C, and 16.9 kcal/mol and 32°C and 12.5 kcal/mol, respectively. Optimum pH values for free tyrosinase and CLTAs are 7 and 6.5, respectively. In addition to that, it was realized that by preparing cross-linked tyrosinase aggregates (CLTAs), the thermostability of tyrosinase was significantly increased. Also, the fact that CLTA particles had nearly same stability during the storage at 4°C and at room temperature was noticed.

Table 1. 4 Optimum conditions for production of CLTAs (Aytar *et al*., 2008)

Enzyme Solution	*Crude Mushroom Extract*
Ammonium Sulfate Concentration	*60% saturation*
Glutaraldehyde Concentration	*2% (v/v)*
Cross-linking reaction	*for 3 hours at room temperature*
Pre-treatment of CLTAs	*Storage in L-lysine solution for overnight*
Washing method	*Filtration*
Particle size reduction	*Mechanical stirring*

1.7 Fungi

In recent years, there has been great interest in exploring the industrial potential and biology of thermophilic fungi. Yet, far more still remains to be explored about these fungi that most represent the most heat-stable eukaryotic organisms.

The microorganism used in this research, *Scytalidium thermophilum*, was a member of fungi which comprise an extremely important and interesting group of microorganisms. They are widespread in nature; grow well in dark and moist conditions where organic material is available.

1.7.1 *Scytalidium thermophilum*

Scytalidium thermophilum, also known as *Humicola insolens*, *Torula thermophila* and *Humicola grisea* var. *thermodia*, is a thermophilic fungus. *Scytalidium*

thermophilum is an important fungus in the production of mushroom compost *(*Toumela *et al.,* 2000*).*

Thermophilic fungi grow massively during he last phase of the composting process, from spores that have survived the pasteurization process. They are believed to contribute significantly to the quality of the compost. The effects of these fungi on the growth of the mushroom mycelium have been described at three distinct levels. First, these fungi decrease the concentration of ammonia in the compost, which otherwise would counteract the growth of the mushroom mycelium. Second, they immobilize nutrients in a form that apparently is available to the growth of mushroom mycelium. And third, they may have a growth promoting effect on the mushroom mycelium (Wiegant, 1992).

The enzymes of thermophilic fungi are generally active at neutral to alkaline pH, and are more thermostable than their counterparts from mesophilic fungi. These properties of enzymes from thermophilic fungi may be explored through a number of novel applications (Ogel *et al*., 2006)

Identification of a bifunctional enzyme, catalase-phenol oxidase (CATPO) from the thermophilic fungus, *S. thermophilum* was identified in previous studies. Sutay et *al*., showed that this enzyme to be a catalase, displaying an additional phenol oxidase activity.

1.8 Scope of the Study

The aim of this study was to investigate the use of cross-linked enzyme (tyrosinase) aggregates (CLTA) obtained from crude mushroom extract for a rapid phenolic content analysis in wines and to compare phenolic characteristics of Turkish red and white wines. In future, these aggregates are going to be incorporated in a biosensor and examined in biosensor applications.

CLTAs were prepared from crude mushroom extracts according to literature data (Aytar *et al*., 2008). Prepared CLTAs using optimum production conditions were tested for their response to different phenolics at different concentrations then reliability and reproducibility of the enzymatic test was examined. After investigation of using CLTAs for phenolic measurement in real wine samples, narrowing of reaction time period was studied. The reactions with the major phenolic compounds present in wine were checked by CLTAs to see the selectivity of CLTAs. Besides, phenolic mixture which mimics the real wine composition was also tested by CLTAs. Finally, total phenolics concentration (TPC) measurement of commercial red wines and white wines produced from Turkish grapes were studied and operational and shelf-life stabilities of CLTAs were examined.

In addition to CLTAs, the phenoloxidase produced by a fungal microorganism, *Scytalidium thermophilum*, was studied in this research to see its capability to oxidize the wine phenolics. Its selectivity towards several wine phenolics was examined. Since this enzyme is really a novel catalase having additional phenol oxidase activity (CATPO), H_2O_2-dependent oxygenation of phenolics with this enzyme was studied to check the possible positive effect of catalase activity (O_2 production) on phenolics concentration measurement.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Mushroom tyrosinase (EC 1.14.18.1), glutaraldehyde (GA) (25 % solution), catechol, L-lysine, acetone, and sulfuric acid, gallic acid, catechin, caffeic acid, p-coumaric acid, malvidin, cyanidin, rutin, myricetin, quercetin, vanillic acid, ferulic acid, syringic acid, Folin and Ciocalteu's Phenol Reagent were purchased from SIGMA (St Louis USA). Ammonium sulfate (AS) was obtained from MERCK (Darmstadt, Germany). 3-methyl-2-benzothiazolinone hydrazone (MBTH) was purchased from Aldrich (Germany). Other reagents were analytical grade and obtained either from SIGMA or MERCK. Mushroom and wines were bought from local supermarket. Ultrapure water was used throughout this research.

2.2 Enzyme Extraction from Mushroom (*Agaricus bisporus*)

Crude enzyme extraction procedures were conducted as described by Aytar and Bakır (2008). Fruit bodies of the mushroom (*Agaricus bisporus*) were obtained from a local supermarket. After removal of stalks, the caps were washed and frozen at 20°C. Then, 100 g of cap tissues were homogenized in 125 ml in 0.1 M, pH 7.0 sodium phosphate buffer, containing 2.5 g of polyvinylpolypyrollidone (PVPP), in a blender (ARÇELİK Rollo K-1350) for 2 min. PVPP can hydrogen bonds to the phenolic compounds present in the extract and prevent phenol-protein interaction (Smith and Montgomery, 1985). The obtained homogenate was centrifuged at 11000 x g for 10 min at 4°C (SIGMA Laboratory Centrifuge 3K18). Supernatant containing the enzyme was filtered and filtrate was used as the crude enzyme extract.

2.3 CLTA Preparation

CLTAs were prepared from crude mushroom extract. CLTA preparation procedures were carried out according to optimized conditions determined in previous studies (Aytar and Bakır, 2008).

Saturated ammonium sulfate (AS) solution was added up to the final concentration of 60% saturation in to a centrifuge tube containing 35U of the enzyme extract. After 5 min of gentle mixing at 4°C, glutaraldehyde (GA) solution was added very slowly to the final concentration of 2% (v/v). This mixture stayed for 3 h at room temperature for the cross-linking reaction. Afterwards, the suspension was centrifuged at 11000 x g for 15 min at 4°C. The recovered pellet was stored overnight in 0.1 M L-lysine solution (prepared with 0.1 M, pH 7.0 sodium phosphate buffer) to react with excess GA present (Tyagi *et al*., 1999). On the next day, CLTAs were recovered and washed via filtration. For this aim, the formed CLTAs were pipetted on to a filter paper (Schleicher & Schuell Grade 589/1 black ribbon) and washed with 0.1 M pH 7.0 sodium phosphate buffer until no enzyme activity was observed in the filtrate. Finally, washed CLTAs were removed from the filter paper, resuspended in buffer and stored at 4°C.

2.4 Maintanence and Cultivation of *Scytalidium thermophilum*

Scytalidium thermophilum was cultivated on YpSs agar plates as described in Appendix A (Cooney and Emerson, 1964). Incubation was performed at 45°C until sporulation, followed by storage at 20°C for maximum 2 months. Inoculation and pre-cultivation procedures were conducted as described by Arifoglu and Ögel (2000).

The enzyme production medium was YpSS except the presence of glucose as a carbon source, instead of starch. In addition, the main culture was supplemented with

copper sulphate. Pre-culture volume was 2% of the main culture volume. After 24 h of incubation at 45°C, the pre-culture was used to inoculate 800 ml of main culture medium (Appendix A). Cultures were incubated in a shaker incubator at 45°C and 155 rpm shaking rate for 5 days. The growth media were filtered through Whatman No.1 filter paper and the filtrate was centrifuged at 8,000 x *g* for 5 min. The supernatant was used as the crude enzyme solution.

2.5 Analytical Methods

2.5.1 Activity Measurement of the Free Tyrosinase

Activity measurement of tyrosinase was conducted spectrophotometrically by measuring the initial reaction rate of catechol oxidation with double-beam UV-Vis spectrophotometer (Thermo Electron Cooperation Evolution 100). As the substrate, 0.1 M catechol solution was prepared in 0.1 M, pH 7.0 sodium phosphate buffer. Enzyme samples were also diluted to the desired end concentrations with 0.1 M, pH 7.0 sodium phosphate buffer. Enzyme, substrate and buffer solutions were preincubated at 35°C. The reaction was started by the addition of catechol solution to the quartz cuvette including the enzyme solution. The volume ratio of enzyme to catechol solution was 1/1. The blank contained buffer instead of enzyme solution. The change of absorbance was recorded at 420 nm in 5 sec intervals for 35 sec.

Initial reaction rates were calculated from the initial linear part of the reaction progress curve. Activity assays were repeated at least for two times.

One unit of the enzyme activity (U) was defined as 1 µmol product formed per min at 420 nm under the given reaction conditions. The extinction coefficient for o-quinone formation from catechol at 420 nm was taken as $\varepsilon_{420} = 3450\ M^{-1}\ cm^{-1}$ (Ögel *et al.*, 2006). Equation 2.1 was used to calculate tyrosinase activity:

$$\text{Enzyme Activity } (\frac{U}{mL}) = (\frac{\Delta OD}{\Delta t\ (sec)})(\frac{1}{\varepsilon})(1000\ \frac{\mu mol}{mmol})\ (2\ \frac{\text{ml reaction mixture}}{\text{ml enzyme solution}})(60\ \frac{sec}{min}) \quad (2.1)$$

Where;

ΔOD = change in the absorbance at 420 nm

Δt =change in the time

ε = extinction coefficient, $\frac{1}{Mcm}$

2.5.2 Activity Measurement of CLTAs

Activity measurement of CLTAs was conducted spectrophotometrically by using a double-beam UV-Vis spectrophotometer (Thermo Electron Cooperation Evolution 100). Appropriate aliquots were taken from the CLTA suspensions and diluted with 0.1 M pH 7 sodium phosphate buffer to the desired end concentrations. 0.1 M catechol in 0.1 M pH 7.0 sodium phosphate buffer was utilized as the substrate solution. Enzyme suspension, substrate and buffer solutions were preincubated at 35°C until they reach to thermal equilibrium. The reaction was started by the addition of catechol solution to the enzyme suspension in 1/1 ratio at 35°C under stirring with a magnetic rod. After 5 sec, 2x1 ml sample was taken from the reaction mixture and filtered through a filter paper (Schleicher & Schuell Grade 589/1 black ribbon) in to a 0.7 ml quartz cuvette. Then the absorbance was recorded at 420 nm. The blank contained buffer instead of enzyme solution. Finally, CLTA activity was calculated by using equation 2.1.

The enzyme recovery in the CLTA was calculated as given in Equation 2.2:

$$\text{Enzyme Recovery} = \frac{\text{total activity of CLEA (U)}}{\text{total free enzyme activity used for CLEA production (U)}} x100 \quad (2.2)$$

2.5.3 Phenol Oxidase Activity Measurement of *Scytalidium thermophilum*

Standard catechol oxidase assay was performed by following the increase in absorbance at 420 nm of the reaction mixture, consisting of 0.5 ml 100 mM catechol solution in 100 mM phosphate buffer (pH 7) as substrate, 0.5 ml enzyme solution at specified concentrations and 1 ml 100 mM phosphate buffer at pH 7 and 60°C was followed. The reference cuvette contained buffer instead of the enzyme. Enzyme activity was measured using the initial rate of the reaction and the extinction coefficient as 3450 M^{-1} cm^{-1} for catechol (Ögel *et al*., 2006) and one enzyme unit was defined as the amount of enzyme required for the formation of one micromole of product per min.

2.6 Enzymatic Phenolic Measurement

2.6.1 Measurement of Phenolics by using CLTAs and Free Enzyme of Tyrosinase

The method of enzymatic oxidation of phenolics to quinones with tyrosinase was selected to measure phenolics concentration. The method is thus based on the oxidation of phenols to quinones as catalysed by the tyrosinase enzyme. Detection is

achieved by measuring the increase in produced quinone amount, which occurs as a result of the enzymatic reaction:

$$\text{Phenol} + O_2 \xrightarrow{\text{tyrosinase}} o\text{-quinone} + H_2O$$

The variation in quinone concentration is determined using a spectrophotometer. 2 ml of different concentrations of standard phenolic solutions in 0.1 mM phosphate buffer was mixed with 20 µl of an enzyme solution. For monitoring the reaction in the visible spectrum, the Thermo Electron Cooperation Evolution 100 spectrophotometer was used. Their absorbances were measured at 350-380 nm. Calibration curves were obtained for several phenolic compounds.

2.6.2 Measurement of Phenolics Concentration by using *Scytalidium thermophilum* Catalase-Phenol Oxidase (CATPO)

2 ml of different concentrations of standard phenolics solutions in phosphate buffer was mixed with 20 µl of an enzyme solution. For monitoring the reaction in the visible spectrum, the Thermo Electron Cooperation Evolution 100 spectrophotometer was used. Their absorbances were measured at 350-380 nm. Calibration curves were obtained for several phenolic compounds.

2.7 Determination of Phenolics Concentration in Wines

CLTA, dissolved free tyrosinase and free CATPO were used for determination of phenolic concentration in various Turkish wines. Red wine samples were diluted by 0.1 M sodium phosphate buffer to set the sample pH to 7. A modified procedure of Besthorn's Hydrazone method (Mazocco *et al*., 1976) was used for the determination of phenolics in wines. The method includes the spectrophotometric measurement of

red complex produced by the interaction (Figure 2.1) between MBTH and the quinones liberated from phenol oxidase activity (Rodriguez-Lopez *et al*., 1994).

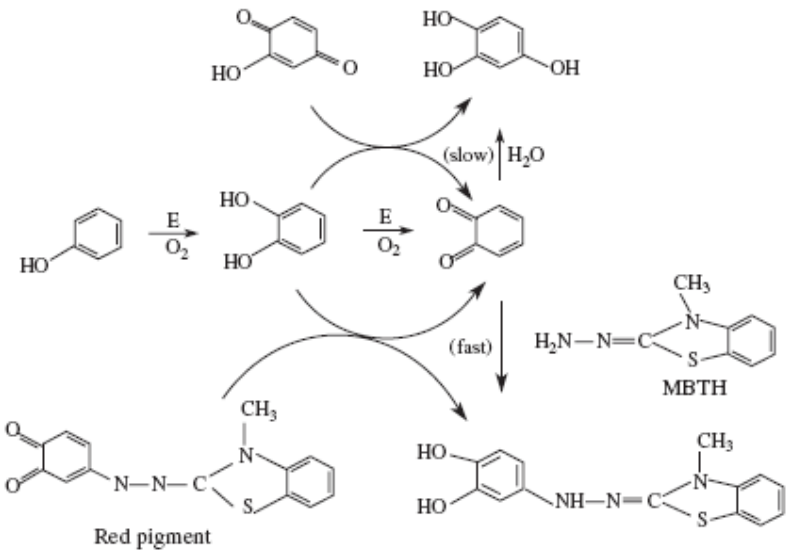


Figure 2. 1 Reaction mechanism of Besthorn's hydrazone method (Rodriguez-Lopez *et al*., 1994)

Different concentrations of catechol solutions were prepared in sodium-phosphate buffer (pH 7). 3.0 ml aliquots in test tubes were placed in water bath at 25 °C. 1 ml MBTH solution was added to the test tubes and enzyme was put into the solutions for specific reaction times. After removing enzyme, 1.0 ml sulfuric acid (5% v/v) was added to stop the enzymatic reaction. 3 ml acetone was added to dissolve the red color complex produced by reaction of MBTH with quinone. Total volume of 6 ml solutions was obtained. After mixing, their absorbances were measured at 495 nm by using a Thermo Electron Cooperation Evolution 100 spectrophotometer and a calibration curve was obtained.

The same procedure was utilized for the measurements of phenolics in red wines except catechol solutions were replaced by diluted wine samples. By using the obtained calibration curve, the phenolic concentration in wines was determined.

2.8 Determination of Operational Stability

Operational stability of the enzymes was determined by measuring its activity for 30 repetitive uses on the same day under the optimum conditions. After each treatment, the CLTAs were washed by buffer solution and sieved through a filter paper until no activity was observed in the collected filtrate. Catechol solution was replaced after each washing step, but the same CLTAs were used.

2.9 Determination of Shelf-Life Stability

Storage stability was investigated for a 50-day period of time by preserving the enzyme solutions in buffer solution at 4 °C when not in use. The activities were measured once in every week.

2.10 Determination of Phenolic Concentration by Folin-Ciocalteau Method

2.10.1 Gallic acid stock solution

In a 100-mL volumetric flask, 0.500 g of dry gallic acid was dissolved in 10 mL of ethanol and diluted to 100 mL with water. It was stored in refrigerator up to 2 weeks.

2.10.2 Sodium carbonate solution

Two hundred grams of anhydrous sodium carbonate were dissolved in 800 mL of water and brought to boiling. After cooling, a few crystals of sodium carbonate was

added and after 24 h, filtered and completed to 1 L. To prepare a calibration curve, 0, 1, 2, 3, 5, and 10 mL of the above phenol stock solution were added into 100 mL volumetric flasks, and then diluted to 100 mL with water. From each calibration solution, sample, or blank, 20 µL were pippetted into separate cuvettes, and to each, 1.58 mL water and then 100 µL of the Folin-Ciocalteau reagent were added and mixed well. After 5 min, 300 µL of the sodium carbonate solution were added, and mixed. The solutions were kept at 20°C for 2 h and the absorbance of each solution was determined at 765 nm. For white wines, 20 µL were added as for the calibration solutions, but in the case of red wines, wines were diluted by 10 first, and then 20 µL were used as samples.

2.11 HPLC Analysis

HPLC analysis was conducted with a Varian ProStar HPLC by using Varian Pursuit C18 (150 x 4.6 m, 5 µm) column at 35°C Analysis was performed with a mobile phase of formic acid/methanol (A/B) at a flow rate of 1.0 mL/min. The mobile phase parameters are listed in Table 2.1. An VARIAN 330 PDA detector operating at a wavelength 280 nm for gallic acid, catechin and syringic acid; 320 nm for vanillic-caffeic, coumaric and ferrulic acid; and 360 nm for rutin, myricetin and quercetine was used. The experiments were conducted in duplicate. All HPLC analysis were performed in METU Molecular Biology and Biotechnology, Research and Development Center.

Table 2. 1 Mobile phase conditions for the HPLC analysis

Mobile Phase	**A:B*** *(For Gallic acid, Catechin, Syringic, Vanillic-Caffeic, Coumaric and Ferrulic acids)*
0 min	0:100
25 min	0:100
60 min	20:80
63 min	20:80
64 min	0:100
70 min	0:100
	A:B* *(For Rutin, Myricetin and Quercetine)*
0 min	20:80
30 min	60:40
31 min	20:80

* A (Formic acid in water, 2.5 % v/v): B (methanol)

CHAPTER 3

RESULTS AND DISCUSSION

The aim of this study was to investigate the use of cross-linked enzyme (tyrosinase) aggregates (CLTA) obtained from crude mushroom extract for a rapid phenolic concentration analysis in wines and to compare phenolic characteristics of Turkish red and white wines. For this purpose, CLTAs were prepared by ammonium sulfate precipitation of tyrosinase from the crude mushroom extract and cross-linking of the formed aggregates by glutaraldehyde using the optimum CLTA preparation conditions determined previously in our laboratory (Aytar and Bakır, 2008).

The preliminary experiments were carried out by employing pure tyrosinase and crude mushroom tyrosinase as the enzyme, and these first efforts comprised the investigation of their response towards several wine phenolics. The next stage involved the examination of their response to different concentrations of phenolic "solutions. Then, the CLTAs were prepared from the crude mushroom extract.

CLTAs of tyrosinase were tested for their response to different phenolics at different concentrations and the reliability and reproducibility of the enzymatic test was also examined. The response of CLTAs for phenolics in real wine samples was also investigated. Afterwards, narrowing of reaction period was studied.

Later, 12 major phenolic compounds of wine were checked by CLTAs of crude mushroom extract to see the selectivity of CLTAs. Besides, a phenolics mixture which mimics the real composition of wine was also tested by CLTAs. Total phenolics concentration (TPC) measurement of 10 commercial red wines and 3 commercial white wines, which are produced from the grapes of the different

geographical areas of Turkey, were studied. Also the consistency of enzymatic test was researched carefully by employing Folin-Ciocalteau method. Operational stability and shelf-life stability of CLTAs were also investigated.

In addition to CLTAs, the enzyme obtained from a fungal microorganism, *Scytalidium thermophilum*, was studied in this research for measurement of phenolic content in aqueous environments. Its selectivity towards wine phenolics was examined. Since this phenoloxidase is really a novel catalase having additional phenol oxidase activity (CATPO), H_2O_2-dependent oxygenation of phenolics with this enzyme was studied to check the possible positive effect of catalase activity (O_2 production) on phenolics concentration measurement.

3.1. Investigation of Tyrosinase Response to Phenolics

Tyrosinase as an enzymatic component oxidizes phenolics forming intensely colored quinones. Using UV-Vis Spectroscopy, the formation of quinone can be observed using their absorption characteristics (Harkensee *et al*., 2006).

In order to observe the response of tyrosinase to catechin, which is the most abundant phenolic found in wines, pure mushroom tyrosinase is used. Figure 3.1 shows the absorption data obtained for the reaction between catechin and tyrosinase.

The oxidation reaction was followed spectrophotometrically at 385 nm, which was reported to be the maximum absorption wavelength of quinone oxidized from catechin (Harkensee *et al.*, 2006). Catechin was completely converted to quinone by oxidation with excess tyrosinase and the formation of quinones led to significant increase in absorption. The escalation in absorption values with the increasing reaction time, as observed in the Figure 3.1, describes the behavior of an expected, typical saturation curve of an enzymatic reaction.

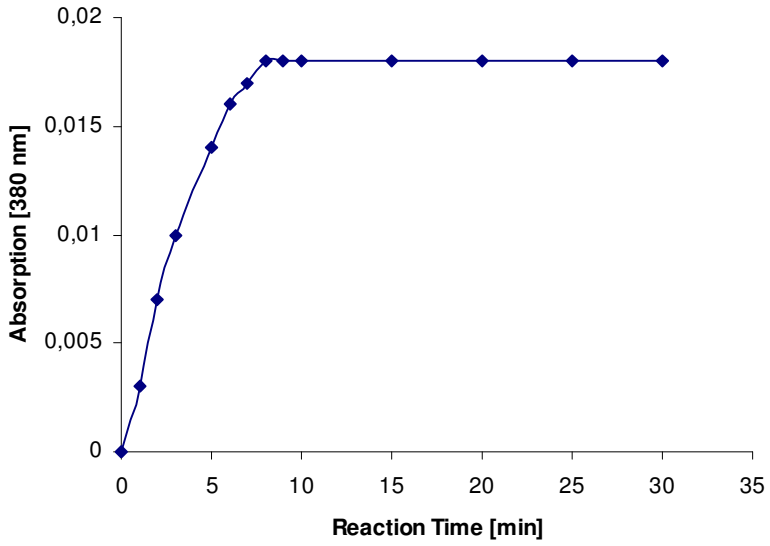


Figure 3. 1 Progress curve for the reaction of a 0,025 mM catechin solution with pure tyrosinase (at 35°C, pH=7, Enzyme activity=270 U/ml)

Later, experiments were carried out to see the response of gallic acid, which is the second most abundant phenolic of wine, by employing both crude mushroom tyrosinase and pure tyrosinase. Crude mushroom tyrosinase was extracted from *Agaricus bisporus*, also known as table mushroom and their oxidation reactions with gallic acid solution was followed at 380 nm, which was reported to be maximum absorption wavelength of quinone oxidized from gallic acid (Harkensee *et al*., 2006). Figure 3.2 shows the absorption data obtained for the reaction between gallic acid and both 2 types of tyrosinase. However, the expected 100% saturation couldn't be obtained even after 70^{th} min for the case of crude mushroom tyrosinase because of the much lower activity of crude mushroom tyrosinase relative to pure tyrosinase. If the reaction would be allowed to continue without any product inhibition or enzyme inactivation, the reaction most probably would stop after about 2 h.

There are 3 different mass transfer limitations in the reaction of CLTAs with phenolics; external and internal mass transfer limitations and reaction rate. Stirring the aqueous environment by a magnetic stirrer helped to overcome the external mass transfer limitations. Therefore, one of the other two limitations controls the conversion. For a free enzyme soluble in aqueous environment, whether pure or crude, product formation is controlled by reaction rate since there is no internal diffusion.

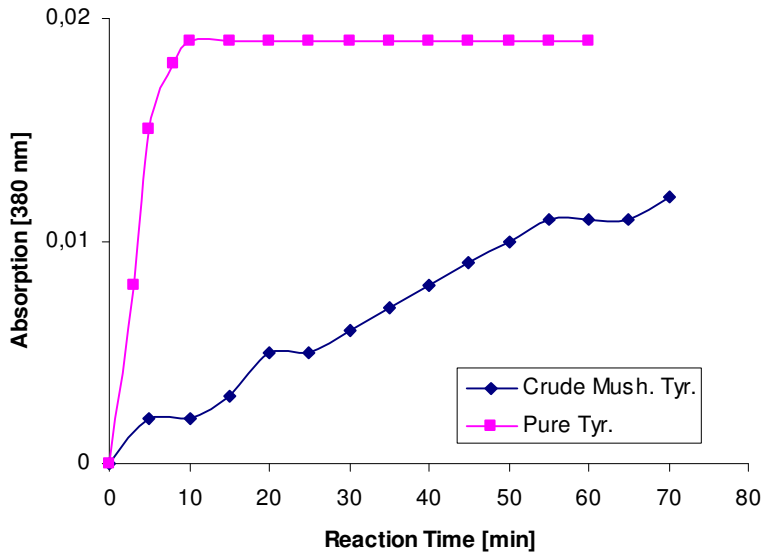


Figure 3. 2 Progress curve for the reaction of a 0.025 mM gallic acid with crude mushroom and pure tyrosinase (at 35°C, pH=7, Enzyme activity=5.4 U/ml)

3.2 Measurement of Gallic Acid Concentration by Tyrosinase

Harkensee, *et al*. (2006) tried to develop a method called spectroscopic enzyme assay for on-site measurement of total phenolics content in grapes and wines. When

catechin, the most abundant phenolics in wine and pure tyrosinase react in the presence of oxygen, they found that 95% of the catechin had been converted into quinone in 15 min. Therefore, they used this finding for the other phenolics measurements and assumed that a 15-min of reaction time is adequate to reach 95% saturation value.

In our experiments, it has been observed that even after 70 min reaction time of gallic acid with crude mushroom tyrosinase, saturation value doesn't reach 100% (Figure 3.2). For this reason, it is hard to estimate the saturation value of gallic acid at 15^{th} min of reaction with crude tyrosinase. In phenolic concentration measurement, an important point is to obtain a linear calibration curve for phenolics at different concentrations. As long as the linearity of the calibration curve is preserved, the saturation level is not so important. Hence, in order to verify whether 15 min is really enough to obtain good linearity of calibration curve for the enzymes, pure tyrosinase, crude mushroom tyrosinase, and CLTA were separately allowed to react with gallic acid solutions for 15 min. The corresponding "absorption/U Enzyme" values obtained spectrophotometrically at 380 nm at different gallic acid concentrations were displayed in Figure 3.3.

It is known that phenolic concentration shows good correlation with the absorption results obtained from the 15-min reaction with tyrosinase. Figure 3.3 shows a linear correlation between the absorption of the enzymatic assay and the gallic acid concentration. Also with regard to the different enzyme activities of different types of enzyme preparations, reliable results were obtained using gallic acid as substrate. For all enzyme types there was a good, linear correlation between absorption results and the total phenolic concentration. However, in the experiments different gallic acid concentration ranges were used for each tyrosinase. There is no special reason for selection of different gallic acid concentration ranges but just because these three experiment sets were performed at different times. The important point here is to obtain linear calibration curves with the use of CLTAs.

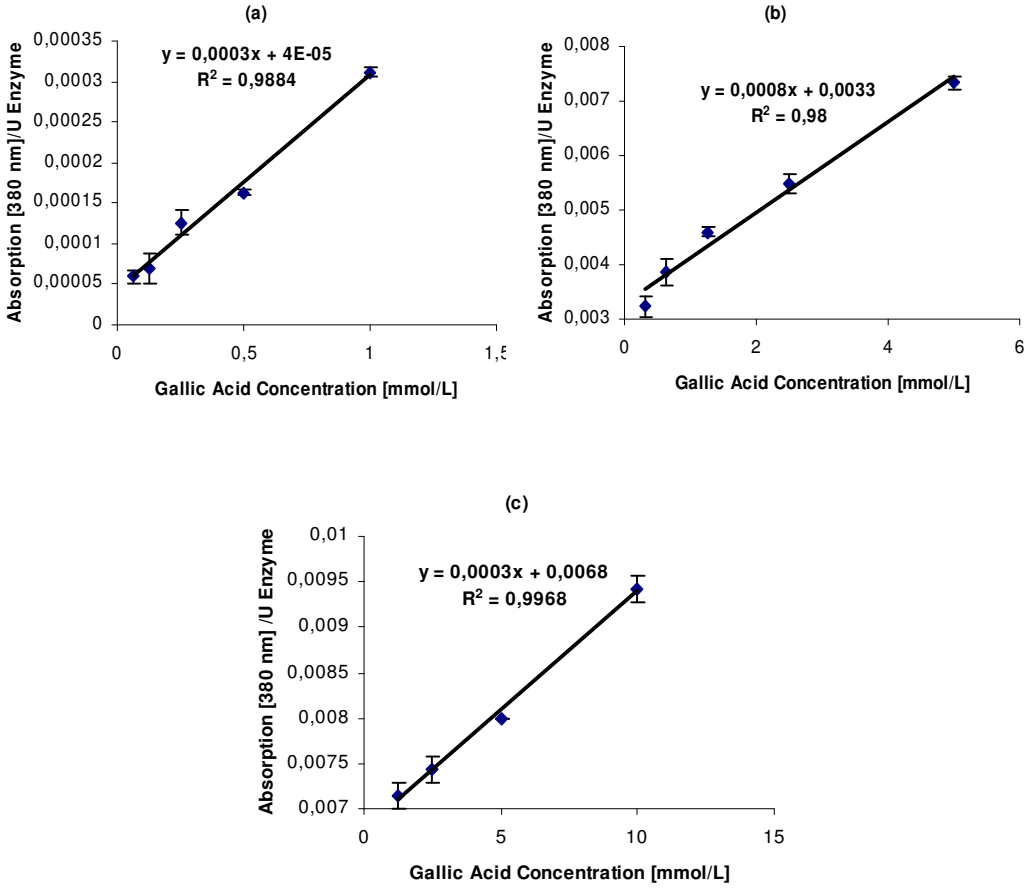


Figure 3. 3 Quinone absorption results of tyrosinase-gallic acid reaction. Also shown are the corresponding results for (a) pure tyrosinase (E.Act=270U/mL), (b) mushroom tyrosinase (E.Act=12.4U/mL) and (c) CLTA (E.Act=3.5U/mL) (at 35°C, pH=7)

For all tested enzyme preparations, total phenolic contents of prepared gallic acid solutions were calculated with enzymatic assay. After 15 min of reaction, absorption spectrum obtained for the samples. Calibration curves were obtained for pure tyrosinase, mushroom tyrosinase and CLTA in a concentration range of 0.06-1.0 mM, 0.30-5.0 mM and 0.30-10.0 mM, respectively. The calculated levels of gallic

acid in the samples are placed in Table 3.1. The concentration range in real wine varies between 7.5 and 15 mM. Since diluted samples were used in phenolic measurements by enzymatic methods, this interval coincides with the ranges used in construction of calibration curves. Therefore, all three types of tyrosinase exhibit suitable ranges for total phenolic content detection.

Folin-Ciocalteau assay is the standard method for the detection of total phenolic concentration in aqueous solutions (Waterhouse, 2001). The reagent is a solution of polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids. It oxidizes phenolates, reducing the heteropoly acids to a blue MO-W complex. The blue colored product is measured at 730 nm. The Folin assay is commonly used in the wine industry worldwide, and the phenolic concentration is expressed in terms of "total phenolics as gallic acid equivalents (GAE)". This unit was chosen because of the varying molecular weights of different phenolics. Since the assay measures all phenolics, gallic acid is normally used as standard due to the fact that it is available as a pure and stable solution. The total phenolic content is always correlated to the signal obtained from a pure gallic acid solution. Thus the results are expressed in gallic acid equivalent (GAE) concentration (Singleton *et al.*, 1999). The results obtained by the Folin-Ciocalteau assay are also shown in Table 3.1.

Table 3.1 shows comparisons between total phenolic concentrations obtained using the Folin-Ciocalteau method and those obtained using the enzymatic assay method in accordance with the real gallic acid concentrations. Real gallic acid concentrations were calculated by dividing weighted gallic acid amount to the volume.

As observed from the Table 3.1, the results of Folin assay validate the results of enzymatic assays. There is relatively good agreement between the Folin-Ciocalteau method and the enzymatic method. The difference in the values obtained by the two

methods does not exceed 6% in sample tested with pure tyrosinase, 12% in sample tested with mushroom tyrosinase and 19% in the sample tested with CLTAs.

Table 3. 1 Total phenolic content of gallic acid solutions

	Pure Tyrosinase	**Mushroom Tyrosinase**	**CLTA**
Enzymatic Assay	0.49 mM±0.04	0.90 mM±0.05	2.5 mM±0.18
Folin-Ciocalteau Assay	0.52 mM±0.01	1.02 mM±0.015	2.1 mM±0.1
Real Concentration	0.50 mM	1.00 mM	2.0 mM
Error (%)	0.06	0.12	0.19

This result may be considered as relatively satisfactory in view of the considerable differences in the two analytical methods used. This amount of difference is also satisfactory for industrial applications.

3.3 Reproducibility of CLTAs

Reproducibility is an important criterion for the reliability of obtained results. Good repeatability of the enzymatic assay with CLTAs was also essential for carrying out stability experiments, which were based on comparison of activity under certain conditions.

Three different batches of CLTA suspensions were prepared under the same conditions. The repeatability of the assay was tested by measuring the conversion of gallic acid into quinone with three different batches of CLTA suspensions using a double-beam UV-Vis spectrophotometer.

As demonstrated in Figure 3.4, calibration curves were obtained for the reaction of gallic acid with all three batches of CLTA suspensions.

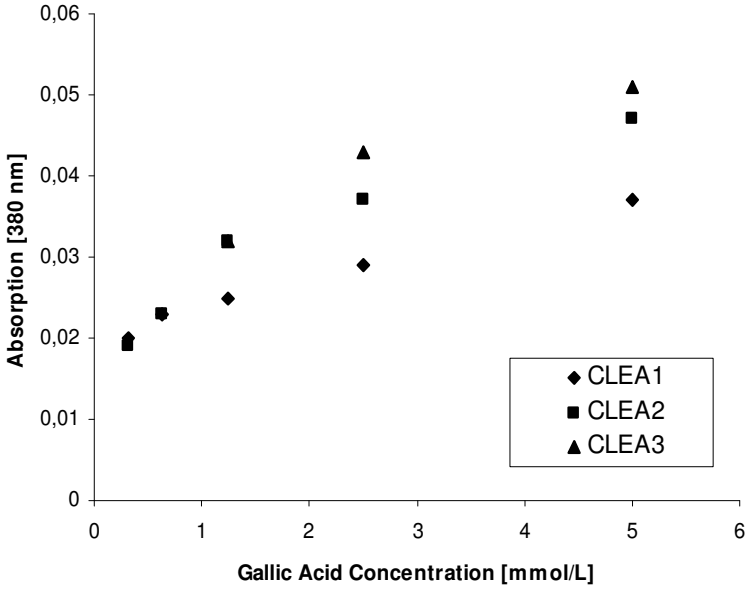


Figure 3. 4 Quinone absorption results of reaction of 3 different batches of CLTAs with gallic acid. (at 35°C, pH=7, E.Activities for CLTA1=12.83 U/mL, CLTA2=22.7 U/mL, CLTA3=38.185 U/mL)

Similar to the results for previous enzymatic test with CLTA, there was a good correlation between the absorption results after a 15-min reaction with all three different batches of CLTA suspensions. Both Figures 3.3(c) and 3.4 show linear correlation between enzymatic assay and the total phenolic concentration, for four different enzymatic tests of CLTA suspensions. Although the calibration curve parameters appear to be different for each batch of CLTAs, their linearities are relatively good. The difference between slopes is possibly due to the difference between used enzyme concentrations. This is not a problem in enzymatic assays. The

important thing is the consistency between the results of calibration curve and quantified samples which are all calculated with the same enzyme.

To determine the accuracy of the results, a gallic acid solution was prepared and its total phenol content was determined by both the enzymatic assay using all three different CLTA suspensions and Folin-Ciocalteau assay. For all tested three different batches of CLTA suspensions, the total phenolics content of gallic acid solution were calculated and the results are given in Table 3.2.

Table 3. 2 Total phenol content measurements of gallic acid solutions with 3 different batches of CLTAs.

	CLTA 1	**CLTA 2**	**CLTA 3**
Enzymatic Assay	1.70±0.18 mM	2.02±0.12 mM	2.20±0.09 mM
Folin-Ciocalteau Assay	2.00±0.01 mM	2.00±0.01 mM	2.00±0.01 mM
Real Concentration	2.00 mM	2.00 mM	2.00 mM
Difference (%)	15.0	1.0	10.0

For all three trials of enzymatic assay with three different preparations of CLTAs, the results of Folin assay confirm the results of enzymatic assays, again. There is clearly good agreement between the Folin-Ciocalteau method and the enzymatic method. The difference of values between the three trials actually does not exceed 15% in the samples tested with CLTAs. Finally, the fact that the measurements have shown a good repeatability allows the conclusion that our enzymatic assay of CLTAs has also a good validity.

3.4 Substrate Specificity of CLTAs

Analysis of the reactions of various wine phenolics with CLTAs was one of the objectives of this study. For this reason, phenolic oxidation reactions were followed for a number of different wine phenolics, and the results were compared with Folin-Ciocalteau assay.

In this section, different phenolic substrates, such as catechin, caffeic acid and *p*-coumaric acid were used to determine the substrate specificity of CLTA activity. The oxidation reactions were followed spectrophotometrically at 385 nm for catechin, 350 nm for caffeic acid and *p*-coumaric acid, which were reported to be the maximum absorption wavelength of quinones oxidized from these phenols (Harkensee *et al*., 2006). Calibration curves were constructed using catechin, caffeic acid and *p*-coumaric acid in a concentration range of 0.13-1.0 mM (37.7-290.0 mg/L), 0.03-1.0 mM (5.4-180.2 mg/L), 0.1-3.0 mM (16.4-492.5 mg/L), respectively. Since, the phenolic amounts of diluted wines that are used to measure phenolic content fall between these ranges (about 1 mM), it is safe to claim that CLTAs are suitable for measurement of phenolic content of wines.

Table 3. 3 A comparison of phenolic ranges in wine and the ones in CLTA test (Total phenolic content for red wine = 7.5-15.0 mM, for white wine = 1.5-2.5 mM).

Phenolics	**Range in Wines (mM)**	**CLTA Test Range (mM)**
Catechin	0.34-0.90	0.13-1.0
Gallic Acid	0.15-0.50	0.30-10.0
***p*-Coumaric Acid**	0.063-0.120	0.1-3.0
Caffeic Acid	0.120-0.200	0.03-1.0

As Table 3.3 exhibits, the phenolic ranges employed in CLTA test method more or less overlap with the actual ranges existing in the wine, a fact which validates the plausibility of the utilized method. Dilution is mandatory in phenolic measurement of wines. Even though the separate ranges of phenolics appear to drop out of the tested interval, that poses no problem since it is the total phenol concentrations that matters, and both red and white wine TPCs stay within these ranges after dilution.

The Figures 3.5, 3.6, and 3.7 imply linear correlations between the absorption values of the enzymatic assay and the total phenolics concentration for all samples. The correlation coefficients were about 0.99 for all three standard phenolics. Considering the different reaction rates of different phenolics, it is safe to claim that the reliable results were obtained using the CLTA assay.

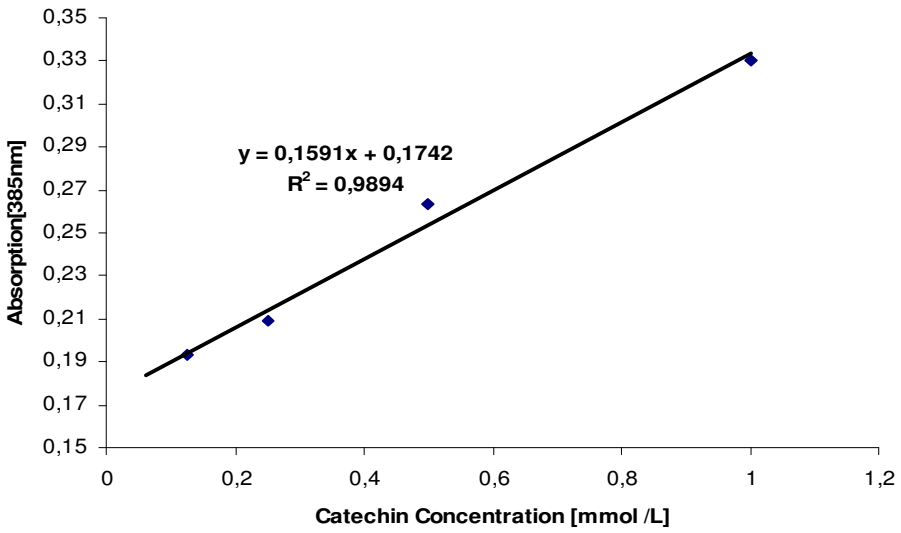


Figure 3. 5 Quinone absorption results of CLTA reaction with catechin solution at different concentrations (at 35°C, pH=7, E.Activity=38.185 U/mL)

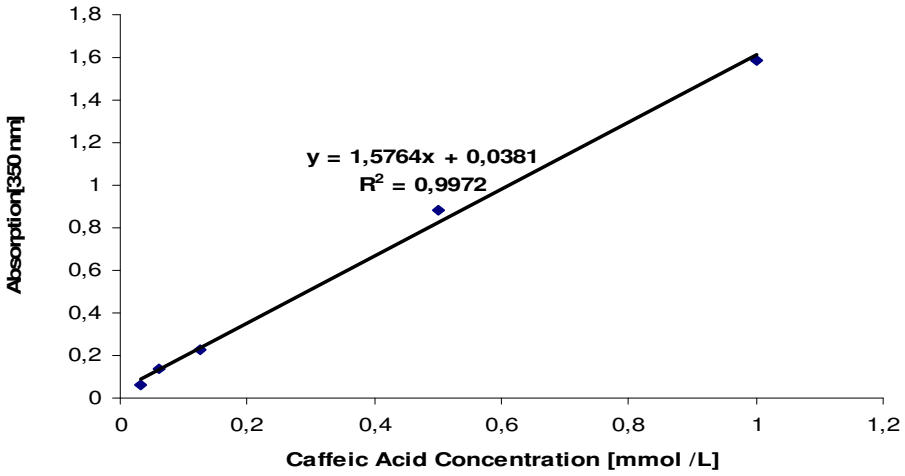


Figure 3. 6 Quinone absorption results of CLTA reaction with caffeic acid solution at different concentrations (at 35°C, pH=7, E.Activity=38.185 U/mL)

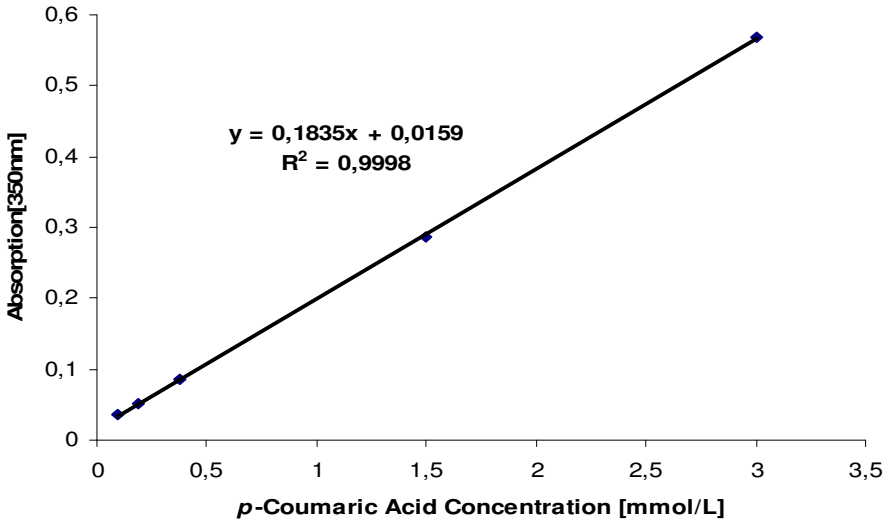


Figure 3. 7 Quinone absorption results of CLTA reaction with *p*-coumaric acid solution at different concentrations (at 35°C, pH=7, E.Activity=38.185 U/mL)

Again, catechin, caffeic and *p*-coumaric acid solutions at different concentrations were prepared and their concentrations were measured by enzymatic assay of CLTA and Folin-Ciocalteau method. For all tested three kinds of standard phenolics, the total phenolics content of catechin, caffeic acid and *p*-coumaric acid concentrations were calculated and the obtained results are given in Table 3.4.

The difference observed between the values of two methods actually does not exceed 1.6% in the tested catechin solution, 4% in the tested caffeic acid solution, and about 13% in tested *p*-coumaric acid solution. Folin-Ciocalteau method and enzymatic method show a relatively good agreement in terms of their responses to the amount of total phenol content.

Table 3. 4 Phenolic Content Measurement Correlation by enzymatic & Folin method

	Catechin Conc.	**Caffeic Acid Conc.**	***p*-Coumaric Acid Conc.**
Enzymatic Assay	0.123±0.016 mM	0.24±0.05 mM	0.85±0.15 mM
Folin-Ciocalteau Assay	0.125±0.002 mM	0.25±0.001 mM	0.75±0.003 mM
Real Concentration	0.125 mM	0.25 mM	0.75 mM
Difference (%)	1.6	4	13

As can be discerned from the above results, the enzymatic assay can be employed to measure a broad range of phenolics of red wine and it provides a rapid and reliable tool for the determination of total phenol content in aqueous environments.

3.5 Phenolic Measurements in Real Wine Samples

CLTAs were tested in diluted wine solutions keeping pH between 6 and 7, without affecting stability and functionality. Dilution factors of wine sample/buffer up to maximum ratio of 1/5 enabled to keep pH within the optimum range, being pH of undiluted real sample far from the values in which the enzyme reach the highest efficiency. On the other hand, the strong matrix effect on the enzyme can be overcome using diluted samples (Montereali *et al*., 2005). For this reason samples were diluted by 1/10. This amount of dilution is also enough to obtain optimum pH which is about 7 for CLTAs.

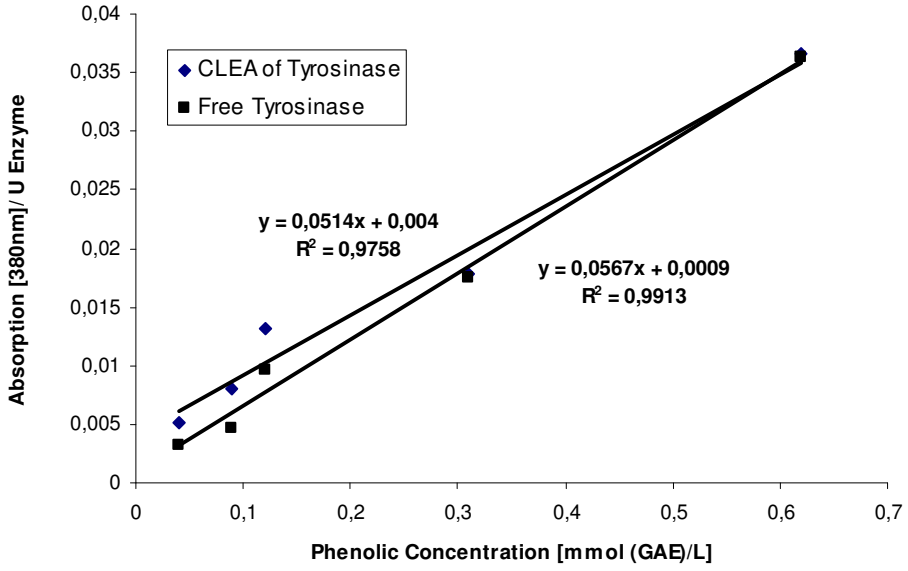


Figure 3. 8 Quinone absorption/enzyme activity results of the enzyme reaction (both free tyrosinase and CLTA obtained from crude mushroom extract) with red wine and the results of the Folin-Ciocalteau assay (at 35°C, pH=7, E.Activity for CLTA=5.8 U/mL, for Free Enzyme=7.4 U/mL)

The absorption after 15-min reaction of diluted red wine solution (1/10 dilution) with both tyrosinase and the CLTA correlated with the results of the Folin-Ciocalteau assay. Examination of the absorption data of reaction of tyrosinase and CLTA with red wine against calculated total phenolic content of red wine with Folin-Ciocalteau method is showed in Figure 3.8.

Similar to the findings for standard solutions, there was a good correlation between the absorption results after a 15-min reaction with both free tyrosinase and tyrosinase CLTA for red wine and the results of the Folin-Ciocalteau assay. Figure 3.8 shows a linear correlation between the absorptions of the enzymatic assays and the calculated total phenolic concentration determined using the Folin-Ciocalteau assay for real sample. The correlation coefficients were R=0.9913 for free tyrosinase and R=0.9758 for CLTA.

The CLTA of tyrosinase showed relatively good signals on real samples comparable to the one in standard solutions.

3.6 Response Time Analysis

Due to introduction of mass transfer limitations and isolation of the enzyme from other deactivating agents found in the aqueous environment, the immobilization process is usually expected to alter the rate of bioreactions (Cao *et al.*, 2003). Besides, these immobilized enzyme aggregates (CLTAs) are planned to be used in the biosensor applications. However, 15-min reaction time for determination of total phenolic content is a poor performance time period for a biosensor measurement. Therefore, the possibility of decreasing this time period to a minimum while still preserving the linearity of calibration curve for standard solutions and real samples was searched.

As can been from Figure 3.9, the conversion of catechin into quinones with respect to time usually happens through a logarithmic pathway. For example, while a 95% conversion is attained at 15 min, only 2 min is sufficient for a 50% conversion. As long as the linearity of the calibration curve is preserved, the saturation level is of no concern. Thus, a logical narrowing of time period should follow a similar pattern. On the other hand, the time period should be long enough to allow the experiment to be carried out properly. Taking these two conditions into consideration, a quasi-logarithmic time decrement was employed, resulting in the following time periods: 15 min, 1 min, and 10 sec.

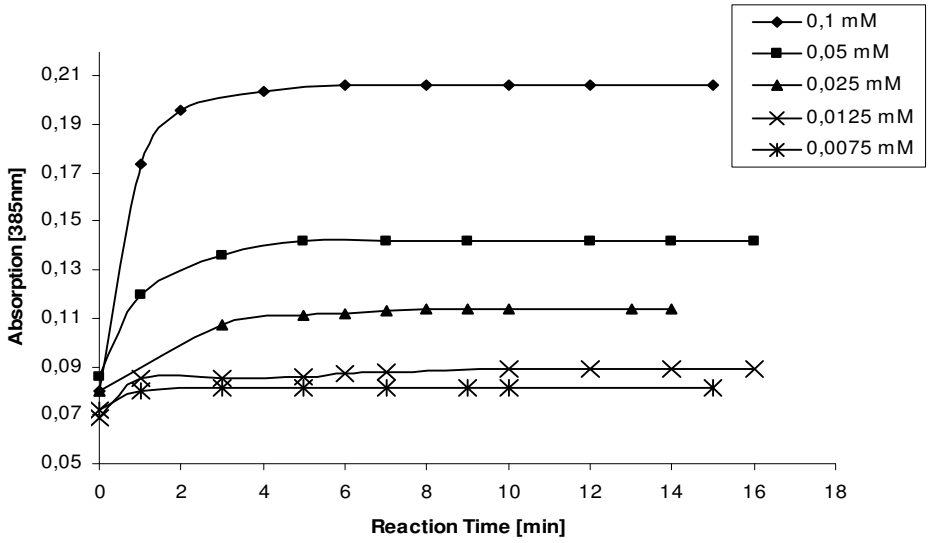


Figure 3. 9 Saturation curves for the reaction of different concentrations of catechin solutions with pure tyrosinase (at 35°C, pH=7, E.Activity=270 U/mL)

For all the experiments performed prior to this point, a 15-min reaction time had been employed for standard and real samples. As the previous data demonstrate,

good correlations between concentration of phenolics and absorption values were always obtained.

On the second step, gallic acid solution and red wine sample are separately allowed to react with CLTA for 1 min. The calibration curves produced from both gallic acid solution (concentration range: 118-1880 mg/L [0.625-10 mM]) and from the wine sample (concentration range: 14-226 mg/L [0.075-1.2 mM]) are exhibited in Figures 3.10 and 3.11, respectively.

At low concentrations of gallic acid solution, particularly for 0.5 mM levels, deviation from calibration curve was observed, probably due to lack of proper diffusion of minute amount of CLTA used. Even, total phenolic content in a typical wine is higher than these values, such as between 7.5-15 mM (1500-3000 mg/L) for red wine or 1.5-2.5 mM (280-470 mg/L] for white wine. On the other hand, there was a better correlation between the absorption results after a 1-min reaction with tyrosinase for wine sample and the results of Folin assay, as shown in the Figure 3.11 below. This finding shows a parallelism with the results of Harkensee, *et al.* (2006).

Having been encouraged with this outcome, the next step was put into execution, i.e., the reaction for 10 sec, which was the shortest time possible to carry out the experiment properly. Therefore, the experiment was repeated with gallic acid and red wine, but the measurement was performed at the 10^{th} sec, this time. Figures 3.12 and 3.13 below display the calibration curves obtained for these two solutions.

The calibration curves obtained for 10 sec of reaction time also provide very good correlations between the concentration and absorption values of both the standard solution (gallic acid) and the real sample (red wine).

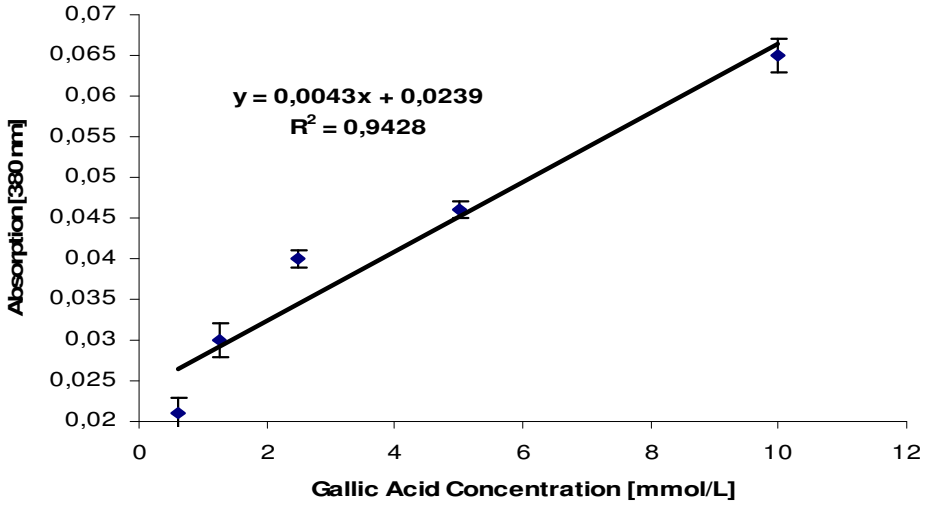


Figure 3. 10 Correlation between absorption of the 1-min CLTA reaction with gallic acid concentration (at 35°C, pH=7, E.Activity= 7.4 U/mL)

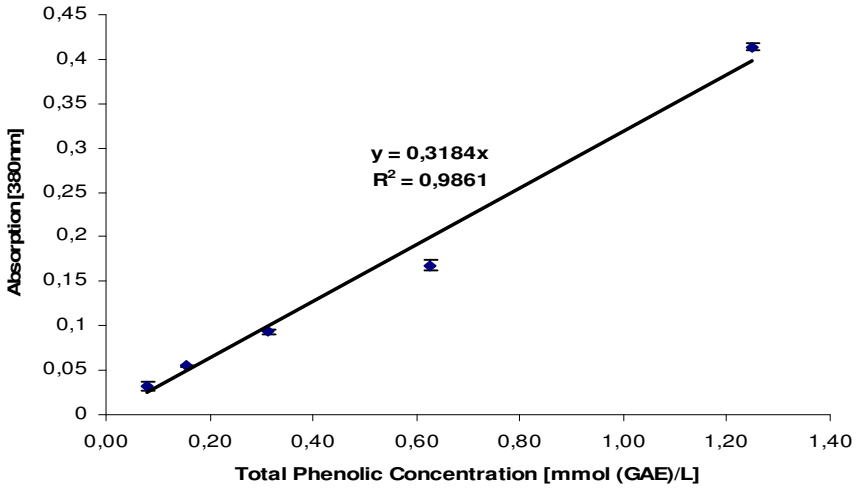


Figure 3. 11 Correlation between absorption of the 1-min CLTA reaction with red wine samples and the results of Folin-Ciocalteau assay (at 35°C, pH=7, E.Activity= 7.4 U/mL)

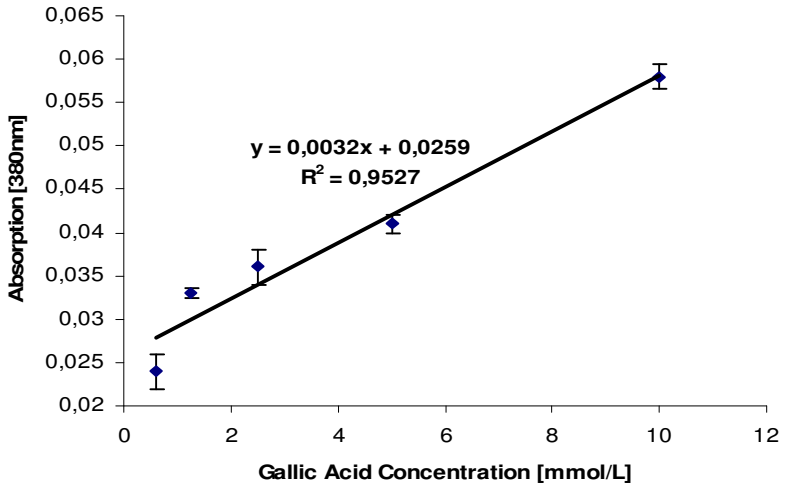


Figure 3. 12 Correlation between absorption of the 10-sec CLTA reaction with gallic acid concentration (at 35°C, pH=7, E.Activity= 7.4 U/mL).

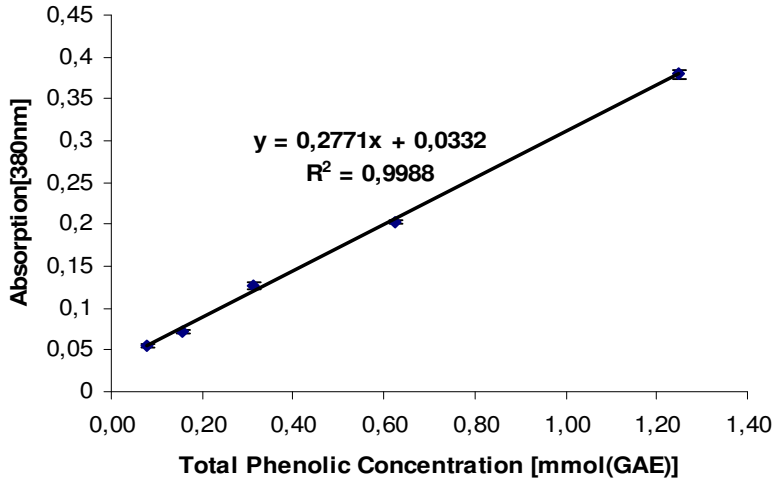


Figure 3. 13 Correlation between absorption of the 10-sec CLTA reaction with red wine sample and the results of Folin-Ciocalteau assay (at 35°C, pH=7, E.Activity= 7.4 U/mL).

Again, the measurement at low concentrations leads to deviation. It can be concluded that CLTAs can give incorrect results at low concentrations. For this reason, higher dilution values than 10 times are not advisable. On the other hand, it is remarkable that the real sample produced smoother lines than the standard solution, even though it may contain many substances. CLTA may respond better to other phenolic acids than gallic acid, or other substances such as ethanol, tartaric acid, etc. may enhance the stability of the results due to the increase in enzyme activity.

Under the lights of these results, the reaction time was established as 10 sec, and used thus at the further experiments. This significant diminution of reaction period from 15 min to 10 sec is very promising regarding the biosensors that will make use of the CLTAs as the detection agent in the future.

Although 10 sec was found to be an effective reaction period to perform TPC measurements, it could trigger certain errors. Because, lower saturation level than 100% was obtained in 10 sec incubation period, measurements should be performed exactly at 10^{th} sec since the reaction did not come to end.

3.7. Selectivity of CLTAs

Spectrophotometry can detect quinones but not the phenolics because of their different absorption characteristics at 350-380 nm. There are 12 phenolics that are most abundant in red wine. The selectivity of tyrosinase was tested by contacting the separate solutions of these phenolics with CLTAs in the presence of oxygen. Tyrosinase normally converts phenolics into quinone. Therefore, by checking the quinone levels through spectrophotometry, it was decided which phenolics of wine had been reacted with the CLTAs. The measurements were performed at 380 nm, since the absorption maxima of quinones of wine phenolics vary within a short range of 350-380 nm to create a significant discrepancy (Harkensee, *et al.*, 2006).

Measurements of phenolics were performed in a synthetic wine matrix dissolving 3 g of tartaric acid in 1 L of 12% v/v ethanol, in order to simulate the composition of real wine samples and a suitable alcohol gradation. Standard solution was prepared and calibration curves were obtained in this synthetic wine matrix for each phenolic.

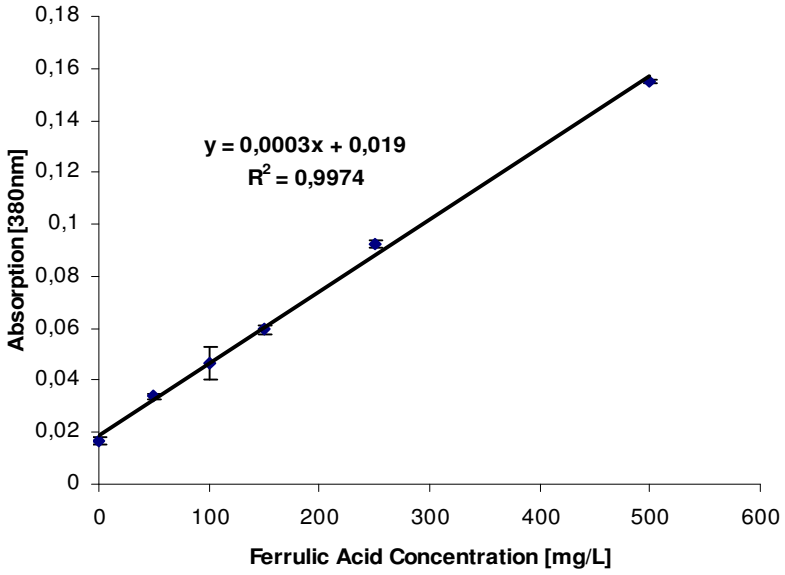


Figure 3. 14 Relationship between ferrulic acid concentration and absorption of the CLTAs reaction with ferrulic acid for 10 sec reaction time (at 35°C, pH=7, E.Activity= 15.8 U/mL)

According to the results, all compounds but the vanillic acid and syringic acid were successfully oxidized by tyrosinase. The calibration curve of ferrulic acid is displayed in Figure 3.14 above as a representative of 10 phenolics, which proved to be responsive, with similar characteristics. Other calibration curves were given in Appendix B.

The calibration curve parameters of these are shown in Table 3.5. With regard to the different reaction rates of different phenolics, reliable results can be obtained using CLTA assay. Anthocyanins, caffeic acid, myricetin and rutin are more reactive than other phenolics. When slope is higher, that means higher conversion of phenolics into quinones occurs. In other words, at 10^{th} sec of reaction, these phenolics reach higher value of saturation than others do. Because phenolic mixture comprised all of these phenolics, the slope of this mixture is like an average of slopes.

As stated in Table 3.5 and as Figure 3.15 depicts, out of all experimented phenolics, only vanillic acid and syringic acid did not give any response.

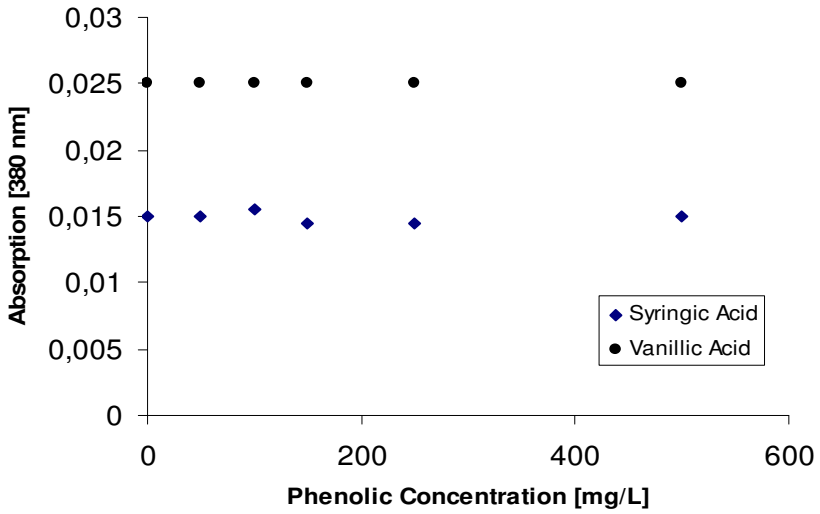


Figure 3. 15 The absorption values obtained for various concentrations of vanillic acid and syringic acid (at 35°C, pH=7, E.Activity= 15.8 U/mL)

Table 3. 1 Calibration curve parameters for enzymatic assays of 12 phenolics of wine, and phenolics mixture (CLTA Act. =15.8 U/mL)

Analyte	Slope	Intercept	r^2	Linear Phenolic Concentration Range (mg/L)	Linear Phenolic Concentration Range (mM)
Gallic Acid	0.00004	0.0194	0.9964	50.0-500.0	0.270-2.700
Catechin	0.00005	0.0147	0.9994	50.0-500.0	0.170-1.700
Caffeic Acid	0.0026	0.0440	0.9984	50.0-500.0	0.280-2.800
p-Coumaric Acid	0.0005	0.0478	0.9999	50.0-500.0	0.310-3.100
Ferrulic Acid	0.00003	0.0190	0.9974	0.0-500.0	0.000-2.600
Vanillic Acid				No response	No response
Syringic Acid				No response	No response
Myricetin	0.002	0.0183	0.9924	10.0-100.0	0.031-0.314
Quercetin	0.00008	0.0489	0.9904	50.0-500.0	0.166-1.660
Cyanidin	0.0066	0.0290	0.9583	1.0-10.0	0.004-0.035
Malvidin	0.0029	0.0223	0.9963	1.0-10.0	0.003-0.030
Rutin	0.0022	0.2553	0.9830	0.0-500.0	0.000-0.750
Phenolics Mixture*	0.0012	0.1004	0.9906	48.0-480.0	0.190-1.920

*Phenolics Mixture concentration is given in Table 3.6

There are clues in the previous literature as to the recalcitrance of these two phenolics against oxidative actions of tyrosinase. Bollag *et al.* (1982) studied the enzymatic oligomerization of vanillic acid and concluded that the oxidative coupling of vanillic acid is not catalyzed by tyrosinase. The same group had also investigated the oligomerization of syringic acid by phenoloxidases in 1981. Various oligomers ranging from dimers to hexamers were determined. They also realized that syringic acid was converted to 2,6-dimethoxybenzoquinone, and this monomer could not be further oxidized by none of fungal enzymes. The nonresponsiveness of syringic acid to tyrosinase could be due to the fact that it is mostly polymerized into many oligomers and the amount of quinone portion could be very low to be detected. Moreover, the generated quinone compound has the *para-* substitution pattern contrary to the usual *ortho-* quinone structure, easily detectable in the spectrophotometer. Montereali *et al.* (2005) also suggest the same possibility on a similar case, while developing a disposable tyrosinase biosensor for the analysis of wine phenolics. When they could not get a response for some phenolics, they ascribed that behavior to the mechanism of tyrosinases, introducing in the first step of reaction pathway a hydroxyl group in *ortho-* position of phenol. The chemical stabilization of the oxidized form (quinone) in *ortho-*, *para-* or *meta-* substitute phenolics varies in their responses. The chemical stabilization of the oxidized form (quinone) in *ortho-*, *para-* or *meta-* substitute phenolics varies in their responses as demonstrated by resonance structures (Yaropolov *et al*., 1995).

When more than one phenolic are present in the same solution, such as the real wine, it is possible that one or more specific phenolics may interfere with the tyrosinase activity. For instance, it is demonstrated that *p*-coumaric acid, quercetin and some other phenolics act as an inhibitor for tyrosinase (Seo *et al*., 2003). For this reason, it was important to check if the detected quinone levels were to be affected by the presence of other phenolic compounds around.

In order to mimic the phenol composition of genuine red wine, a phenolics mixture was prepared with reference to the values of three separate HPLC results of real red wines previously published in the literature (Tarola *et al.*, 2007; Gomez-Alonso *et al.*, 2007; Garcia-Falcon *et al.*, 2007). The composition of the mimic mixture is given in Table 3.6. A rough estimation of phenolic composition of this hypothetical mimic mixture was done by presuming that all phenolic composition is formed from these 12 phenolics. This mixture was tested with CLTA to correlate the quinone level with the phenolics concentration, and the resulting parameters of the experiment were placed in the last row of Table 3.5. As these parameters imply, the phenolics mixture also shows good linear correlation as other separate standard solutions (Figure 3.16).

Table 3. 5 The composition of the mimic phenolics mixture

Phenolics	**Concentration(mg/L)**	**(%)**
Catechin	156.5	31.3
Gallic Acid	78.0	15.6
Caffeic Acid	52.0	10.4
Vanillic Acid	52.0	10.4
Syringic Acid	36.5	7.3
Ferrulic Acid	26.0	5.2
p-Coumaric Acid	26.0	5.2
Quercetin	26.0	5.2
Rutin	15.5	3.1
Myricetin	10.5	2.1
Cyanidin	10.5	2.1
Malvidin	10.5	2.1
Total	500	100.0

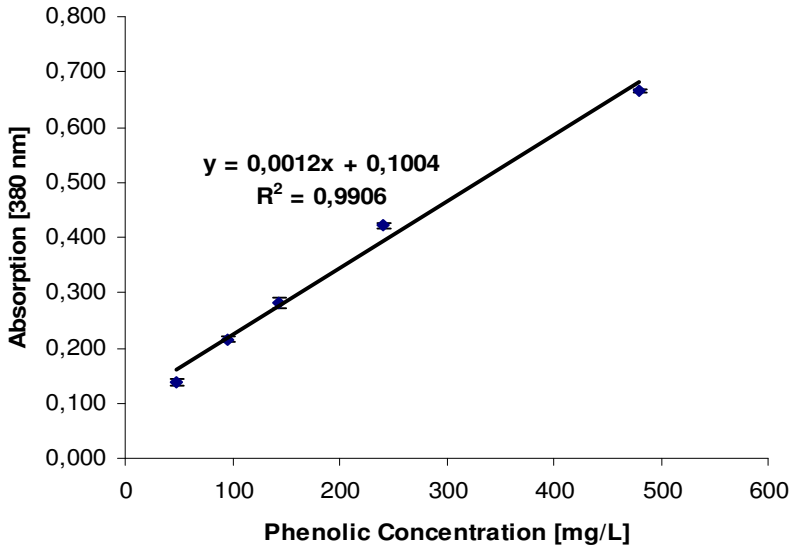


Figure 3. 16 Absorption results of reaction of CLTAs with a phenolic mixture at different concentrations (Reaction Time=10 sec, at 35°C, pH=7, E.Activity=15.8 U/mL)

A genuine red wine (produced in 2007) was also put under the same reaction with CLTAs and the absorption values were recorded. This absorption reading was put into the calibration curve equation of phenolics mixture, and the corresponding phenol concentration was calculated. On the other hand, Folin assay was applied to the same red wine to find total phenol concentration. The value obtained from Folin assay (2085 mg/L) verified the accuracy of enzymatic assay result (2188 mg/L), thus the reliability of the phenolics mixture's calibration curve.

3.8. Determination of Total Phenolics Content in Turkish Red Wines

Turkish red wines were used in the analysis of total phenolic content measurements. Total phenolic compounds in Turkish wines are reported as 2000–3000 mg/L

(Karakaya *et al.*, 2001). Phenoloxidases (PO) act on –OH groups on phenolic compounds. The total –OH groups were obtained via activity determination of enzyme in red wine.

In determination of total phenolic content in Turkish red wines, Besthorn's Hydrazone Method was employed. In this method, MBTH interacts with the quinones produced by the enzyme to yield red products instead of brown color pigments in the absence of the color reagent (Ortega *et al.*, 1993).

Figure 3.17 shows different concentrations of catechol solutions after reacting with CLTAs in the presence of 0.3% MBTH solution. As can be seen from the photograph, the most concentrated solution gives the most reddish color. It was just as expected, because the red-colored compounds insinuate the existence of quinone-MBTH complex amount; and it was most abundant in the first tube, while it was non-existent in the last tube where no color change was observed.

The application of the MBTH-coupled assay to the determination of total phenol amount of wines with tyrosinase has various properties and advantages over other methods. First, the MBTH assay of tyrosinase is approximately 8-times more sensitive than the commonly used methods, employing phenolics as substrate. Besides, the method can be employed to assay a high variety of phenolics. It is useful for phenolics with both cyclizable and non-cyclizable side-chains. Moreover, the presence of MBTH in the reaction is reported not to modify the kinetic constants of tyrosinase on its phenol substrates. One other advantage is that it may be quickly and routinely performed with standard laboratory spectrophotometers. This method provides a high molar extinction coefficient in the visible region of the spectrum, where tyrosinase, monophenols, and o-diphenols do not exhibit absorbance maxima. The method is effective even when micro amounts of tyrosinase is present in the environment (Rodriguez-Lopez, *et al.*, 1993).

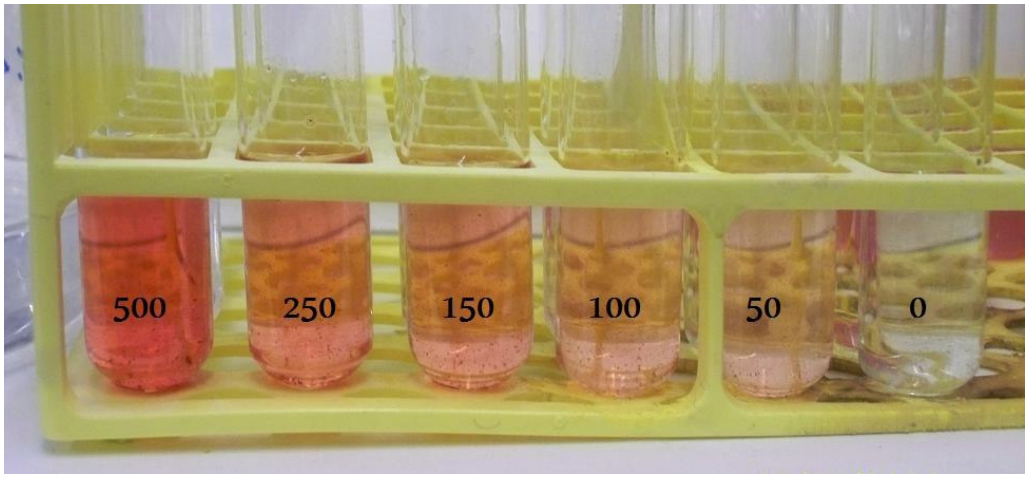


Figure 3. 17 The appearance of test tubes containing different concentrations of catechol after reacting with CLTAs in the presence of MBTH. (Reaction Time=10 sec, pH=7, T=35°C, E.Activity= 25 U/mL)

It has become apparent that the phenol oxidation by immobilized tyrosinase is caused only by an enzymatic action. Although the color of reaction solution was changed to red by immobilized tyrosinase, the immobilized tyrosinase itself was not colored. This finding suggests that phenol oxidation products (such as quinones) do not react with immobilized tyrosinase and do not absorb on the enzyme.

Ten commercially available red wines produced from different grape cultivars grown in different regions of Turkey were analyzed in terms of their total phenol contents by both Folin-Ciocalteau method and enzymatic method of CLTAs. The pH values of untreated real samples are usually far from the values in which the enzyme reaches the highest efficiency (Montereali, *et al.*, 2005). On the other hand, too much dilution would lead to different problems, such as dissolved oxygen depletion. Hence, the red wine samples were diluted at a ratio of 1/10, before the enzyme was introduced in order to keep the pH within the optimum range.

Table 3. 1 Analytical results of ten commercial Turkish red wine samples

Sample	Grape Cultivar	CLTA Assay (mg/L)	Folin-Ciocalteau Method (mg/L)	Difference (%)
Brand I	Elazığ-Bogazkere (2005)	2483	3055	18.72
Brand II	Bozcaada-Karalahna (2005)	2683	2600	3.18
Brand III	Öküzgözü/Boğazkere (2005)	2369	2318	2.20
Brand IV	Ege-Şiraz/Kalecik Karası (2006)	2165	2209	2.02
Brand V	Boğazkere/Gamay/ Cinsanet (2006)	1996	2136	6.55
Brand VI	Elazığ-Öküzgözü (2006)	2665	2136	24.72
Brand VII	Trakya-Gamay (2005)	1969	2118	7.03
Brand VIII	Karasalkım/Papazkarası (2006)	1974	1928	2.37
Brand IX	Karasalkım/Gamay/ Kalecik Karası (2006)	1569	1900	17.42
Brand X	Kalecik Karası (2006)	1710	1700	0.59

Dilution also helps to overcome the strong matrix effects of real wines on the enzyme. The results obtained for both mentioned methods are displayed in Table 3.7.

Yıldız *et al.* (2005) stated that the results for phenolic determination by using free enzyme give very small values when compared with the actual concentrations. It is known from the literature that benzoates act as inhibitors for free PO. So, PO is inhibited by benzoates, found naturally in wines, before it completes enzymatic reactions. However, the results of our experiments did not differ too much from the real values obtained by Folin-Ciocalteau assay. This was a good demonstration of several advantages of CLTAs over the free enzyme methods.

Previous studies suggest that immobilized PO was protected by the matrix entrapping it and was not affected by the inhibitors found in wines (Lopez *et al.*, 2001, Kıralp *et al.*, 2003, Kıralp *et al.*, 2004, Kıralp *et al.*, 2006). Probably, the cross-linked structure of enzyme aggregates also brought into being a similar effect.

Brand I and Brand II have the highest phenolic content as displayed in Table 3.7. This might be emanating from the peculiar traits of Boğazkere and Karalahna grapes, or rather from the aging factor. A few of the samples exhibited slight deviations from the real values, which can be attributed to the relative selectivity of tyrosinase towards the oxidation of certain phenolics. The difference between mechanisms of methods can also lead to this difference. Higher amount of inhibitors in wines can decrease the activity of CLTAs.

3.9. Determination of Total Phenolic Content in Turkish White Wines

The assay procedure explained in Section 3.8 was applied in the same way for white wines. Three commercially available white wines produced from grapes coming from different regions of Turkey were analyzed in terms of their total phenol contents by both Folin-Ciocalteau method and enzymatic method of CLTAs.

The only difference is that white wines were not diluted because of their low phenolic content. Besides, their pH value doesn't create a problem for CLTAs. The results obtained for both mentioned methods are displayed in Table 3.8.

The results of enzymatic assay are found to be comparable to the values of phenolic compounds obtained by Folin-Ciocalteau method in Turkish white wines. According to Folin-Ciocalteau method Brand XIII contains the most phenolic amount among three white wines. This result was also confirmed by CLTAs.

Phenolic amount in white wines are much lower than in red wines. The main reason is that grape juice used to make red wine contains skins, seeds, and stems. This is significant for the following reason: leaving juice to mix together with the woody bits (known as maceration) causes the finished product to contain tannins. Tannins can lend a wonderful complexity to a red wine. In addition to that most of the phenolic substances are found in the seeds and skins of the grapes. As a general rule, red wine phenolics are heavier and more complex than white wines. White wines don't have most of the anthocyanins which give the red color to wines.

Table 3. 1 Analytical results for three commercial Turkish white wine samples.

Sample	Grape Cultivar	CLTA Assay (mg/L)	Folin-Ciocalteau Method (mg/L)	Difference (%)
Brand XI	Pamukkale-Emir (2005)	280	252	11
Brand XII	Güney Denizli-Chardonnay/Narince/Sultaniye (2006)	300	259	16
Brand XIII	Ege-Sultaniye/Semillion (2006)	380	350	9

3.10 HPLC Results

The phenolic content analysis of 10 commercially available red wines produced from grapes of Turkey was also performed by HPLC technique. Although it is known that red wine samples have much higher number of different phenolic substances, analysis was performed for only 10 different wine phenolics which are known as the most abundant ones in red wines. The results of phenolic compounds for selected red wines are shown in Table 3.9. Gallic acid and catechin turned out to be the most abundant phenolics in red wines as expected from literature data.

Moreover, the data may provide us with some information on the wines and the grapes of different regions. Brand IX, for example, is produced from Karasalkım/Gamay/Kalecik Karası grapes, and as can be seen from Table 3.9, the tested 10 phenolics comprise almost all of its phenolic content. However, for Brand VI, these 10 phenolics comprise about 20% of its total polyphenol content. According to the Robichaud *et al.* (1990), the monomeric catechins are bitter and astringent. However, in the polymer, the bitterness is minimal, but the astringency remains. Taking this finding into consideration, we can expect Brand IV to be the most astringent without consulting to an expert. Then comes Brand VIII and Brand II, and these three are probably are more bitter than the others. The results also imply that Kalecik Karası, Şiraz and Karalahna grapes are rich in catechin amount.

Coumaric acid, on the other hand, is discovered by previous studies to result in substantial variations in the color of the wine (Bloomfield *et al.*, 2003, George *et al.*, 2001). Moreover, *p*-Coumaric acid has antioxidant properties and is believed to reduce the risk of stomach cancer by reducing the formation of carcinogenic nitrosamines (Kikugawa *et al.*, 1983, Ferguson *et al.*, 2005). Brand IX and Brand II, thus, seem more promising in terms of preventing stomach cancer.

Table 3. 1 HPLC values of mean concentrations of 10 phenolic compounds in selected 10 Turkish red wines (mg/L)

Grape Cultivar	Gallic Acid	Catechin	Syringic Acid	VanillicAcid /CaffeicAcid	Coumaric Acid	Ferrulic Acid	Rutin	Myricetin	Quercetin	Total[b]
Elazığ-Bogazkere (2005)	55.4	126.6	39.0	439.3	50.8	11.1	27.3	7.4	24.6	3055
Bozcaada-Karalahna (2005)	127.6	170.3	18.7	110.7	484.7	59.6	12.7	12.3	31.4	2600
Öküzgözü/Boğazkere (2005)	125.5	50.5	11.9	126.6	14.4	1.6	10.2	4.4	6.2	2318
Ege-Şiraz/Kalecik Karası (2006)	66.6	242.1	32.0	198.0	35.7	1.8	26.5	10.5	23.1	2209
Boğazkere/Gamay/ Cinsanet (2006)	41.5	113.2	27.4	86.2	17.3	2.8	27.2	6.7	12.1	2136
Elazığ-Öküzgözü (2006)	63.2	105.4	20.3	40.3	9.9	0.5	15.3	21.2	38.9	2136
Trakya-Gamay (2005)	46.7	166.6	33.7	59.6	16.8	1.4	22.8	6.6	15.3	2118
Karasalkım/ Papazkarası(2006)	59.1	181.1	30.4	154.9	18.6	2.4	28.1	12.4	29.3	1928
Karasalkım/Gamay/ Kalecik Karası (2006)	75.0	118.1	32.7	149.9	950.3	81.9	33.9	12.1	29.8	1900
Kalecik Karası (2006)	48.0	154.2	23.5	128.5	18.3	4.5	25.1	16.1	28	1700
Literature Values[a]	13.0-73.3	3.1-179.4	0.4-10.0	1.0-23.0	0.5-4.7	0.3-4.0	2.7-10.0	0.3-12.2	1.7-15.1	1500-3000

[a]Average values of phenolic concentrations obtained from literature (Tarola *et al.* 2007, Garcia-Falcon *et al.* 2007, Gomez-Alonso *et al.* 2007, Castellari *et al.* 2002, Woraratphoka *et al.* 2007).
[b]Total phenolics content measured by Folin-Ciocalteau Method

These two brands are also rich in ferrulic acid, which is a strong antioxidant and helps the immune system of the body (Kong *et al.*, 2007). Gallic acid, like other phenolics, has also antioxidative properties.

According to HPLC-results, the wine that has the highest amount of myricetin and quercetin is produced from Öküzgözü grapes (Brand VI). Price *et al.* (1995) have shown that red wines coming from lower yielding vines with better sun exposure of the fruit have at least three-fold higher levels of flavonols, suggesting that the levels of these compounds in grapes may be a useful indicator of grape sun exposure and perhaps quality. The fact that Boğazkere and Gamay grapes were used in the production of wines containing the lowest myricetin and quercetin levels may augur that these grapes were grown on rather fecund vines, thus allowing to lesser sunlight exposure.

Quercetin is a flavonoid with a wide range of biological activities. It mainly occurs in plants as glycosides, such as rutin (quercetin rutinoside) in wine. Quercetin and rutin are reported to be used in many countries for blood vessel protection and are ingredients of numerous multivitamin preparations and herbal remedies (Sahelian, 2007).

3.11 Operational Stability of CLTAs

The major disadvantage in the enzymatic measurement of phenolics is the inactivation of the enzyme by quinones, and thus, no reusability. Immobilization of tyrosinase was investigated in an effort to overcome these problems. The CLTAs show promising results, and in future the method may be successfully incorporated into a biosensor to detect phenolics contents in aqueous environments. A desirable biosensor should be able to maintain its effectiveness through many repetitive usages. So as to elucidate the ability of immobilized tyrosinase to be used repeatedly,

catechol oxidation on repeated treatments was carried out. The CLTAs were washed by buffer solution and sieved through a filter paper after each treatment until no activity was observed in the collected filtrate. Catechol solution was replaced after each washing step, but the same CLTAs were continued to be used. Figure 3.18 depicts the results of 30 repetitive runs with the same CLTAs. The absorption values fluctuated a little, but never ceased to stay within a small range, thus proving the stability of the method convincingly.

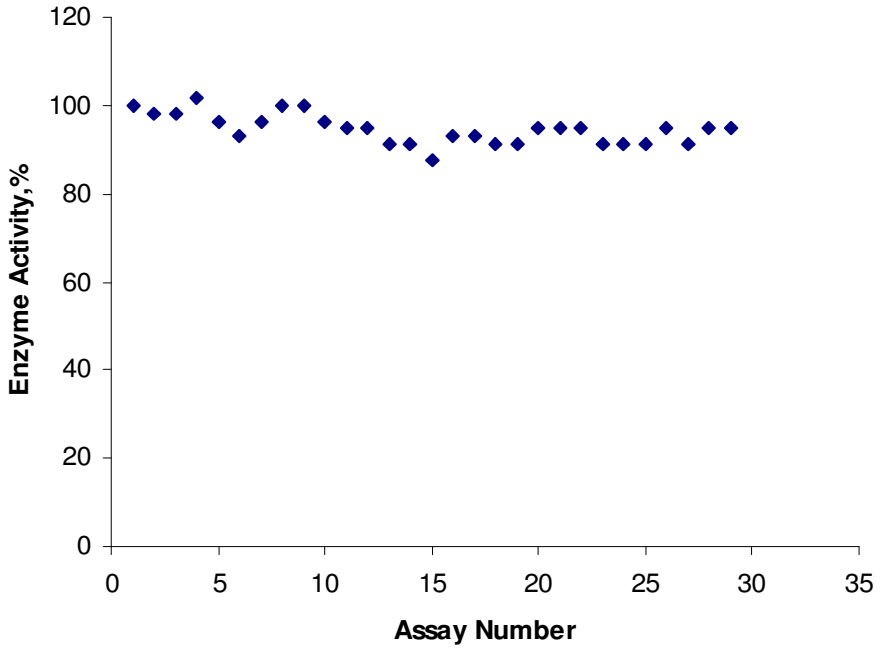


Figure 3. 18 Operational Stability of CLTAs (at 35°C, pH=7, E.Activity=25 U/mL)

3.12 Shelf-Life Stability of CLTAs

Enzymes can easily lose their catalytic activity and denatured, so careful storage and handling of enzymes are important. The CLTAs were stored in buffer solution at to ease handling and to prolong the shelf-life of the enzyme. As Aytar *et al.*, (2008) indicated storage temperature (room or refrigerator temperature) had no effect on the CLTAs activity. So, stability of CLTAs was examined by means of time.

For the shelf life determination of the CLTAs, the activities of CLTAs were checked every week throughout 50 days. Figure 3.19 shows the shows the effect of storage time on the activity of the immobilized tyrosinase. The rate of loss of activity of the CLTAs was very low for first days, retaining 98% of the initial activity after 15 days. After 15th day, enzyme activity decreased (not much however) to 90% and stayed constant up to 50th day. A long shelf-life and acceptable stability for enzyme is present in the form of CLTAs.

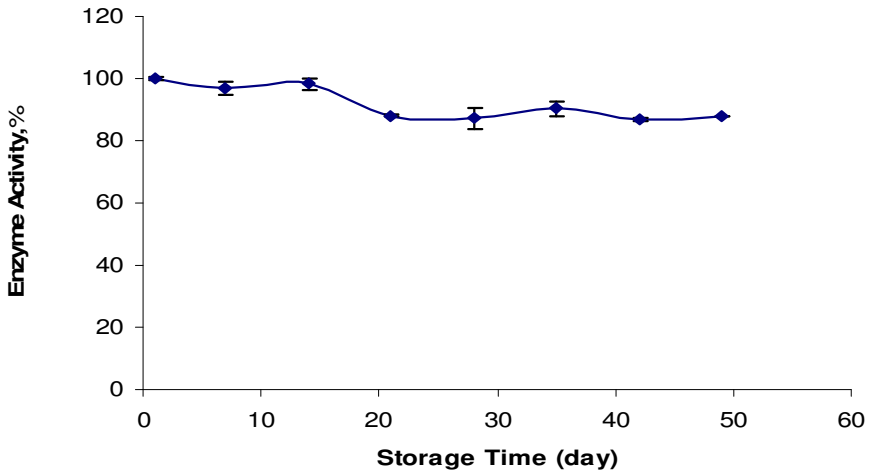


Figure 3. 19 Shelf-life of CLTAs (at 35°C, pH=7, E.Activity=8.75 U/mL)

3.13 Measurement of Phenolic Concentration by *Scytalidium thermophilum* Phenol Oxidases, CATPO

Ögel, *et al.*, (2006), affirmed that *Scytalidium thermophilum* produces a single major extra-cellular phenol oxidase on glucose-containing medium. It was declared that the extracellular phenoloxidase behaves more like a laccase due to oxidation of the amino-aromatic compound ADA, as suggested by Rescigno *et al.* (1997). However, according to Sutay *et al.* (2008), culture supernatant showed catechol oxidase activity but no laccase-like activity since the pure enzyme oxidizes neither syringaldazine nor ABTS which are accepted as unique substrates for true laccase activity (Burke and Cairney, 2002). In addition to that, it was confirmed that tyrosine is not hydroxylated by enzyme, showing absence of the cresolase activity. Thus, the enzyme shows neither tyrosinase like nor laccase like activity.

In 2008, Sutay *et al*. after purification, characterization, and amino acid sequencing of this enzyme, revealed that this enzyme is actually a catalase, displaying an additional phenol oxidase activity. It was described as a bifunctional enzyme, catalase-phenol oxidase (CATPO) from the thermophilic fungus*, S. thermophilum*.

Catalases (EC 1.11.1.6) are metalloenzymes that catalyze the degradation of hydrogen peroxide (H_2O_2) to dioxygen and water (Goldberg and Hochman, 1989). This function is important in a phenol oxidation reaction because oxygen concentration in the environment as high as possible. It was known that increase in oxygen concentration will result in increase in product in a phenol oxidation reaction if oxygen concentration is low. Because our phenolic measurement method is based on the measurement of phenolic concentration, presence of O_2 at high concentration can improve the method.

It was found that certain phenolic acids, specifically gallic acid and tannic acid, induce the expression of the enzyme (Sutay *et al*., 2008). Under the light of these information, to see the selectivity of this CATPO against other wine phenolics, various phenolic substrates; catechin, p-coumaric acid, caffeic acid, vanillic acid, syringic acid, quercetin, ferrulic acid, rutin were used to examine the substrate selectivity of *S.thermophilum* catechol oxidase enzyme.

Both syringic and vanillic acids gave no response, as observed with tyrosinase. Figure 3.20 displays the absorption values for reaction of CATPO with vanillic and syringic acids. As a result, it can be claimed that, syringic and vanillic acids can't be hydroxylated by the enzyme produced from *S.thermophilum*.

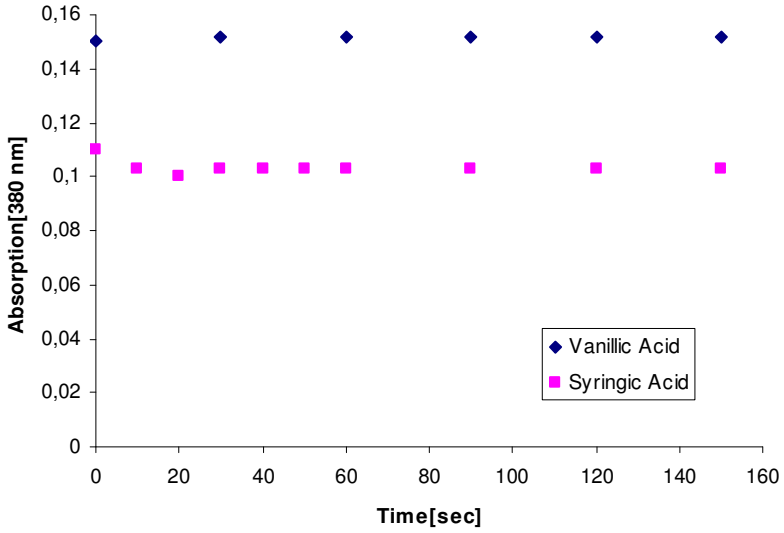


Figure 3. 20 The absorption values obtained for the reaction of vanillic acid and syringic acid with CATPOs. (Reaction Time=10 sec, at 60°C, pH=7, E.Activity=40 U/L)

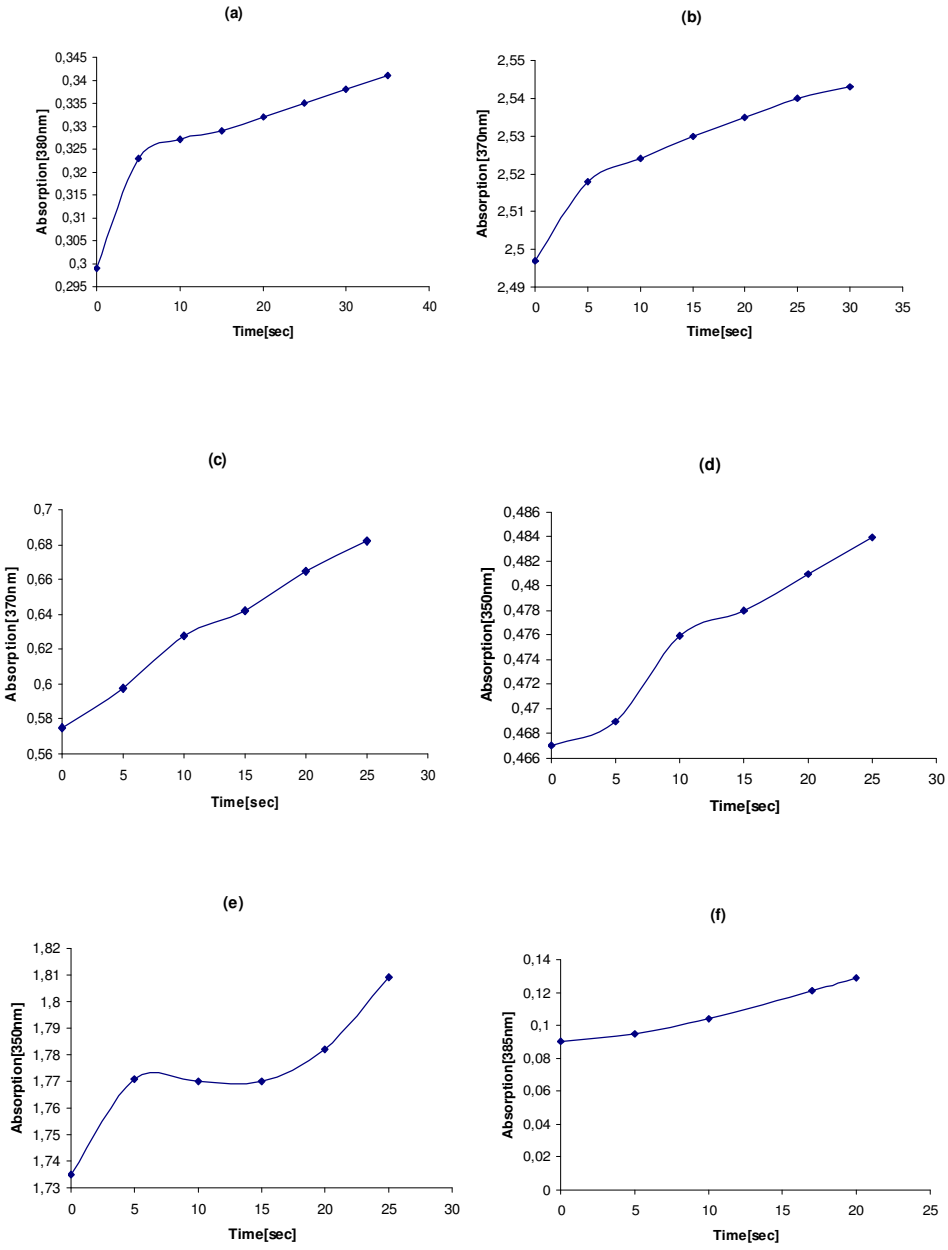


Figure 3. 21 Saturation curve for reaction of 500 mg/L phenolics with CATPO withoutt H_2O_2 (a) ferrulic acid (b) rutin (c) quercetin (d) p-coumaric acid (e) caffeic acid (f) catechin (at 60°C, pH=7, E.Activity=40 U/L)

Saturation curves of 6 phenolics (catechin, caffeic acid, p-coumaric acid, quercetin, ferrulic acid, rutin) are displayed in Figure 3.21, which proved to be responsive, with similar characteristics. As can be seen from the Figure 3.21, formation of quinones from phenolics, demonstrate a rapid increment in 10 sec. The differences between curves can be explained as a result of impurities in enzyme solution. Although there are some differences between saturation curves, it can be concluded after 10^{th} sec the increment rate decreases with a high proportion. Thus, a reaction time of shorter than 10 sec should be chosen for further experiments which can cause problems in application. To increase this period, enzyme solution can be diluted more and reaction rate is decreased. Although, more research should be performed to optimize this incubation period, 10 sec was chosen as incubation period for all other further experiments.

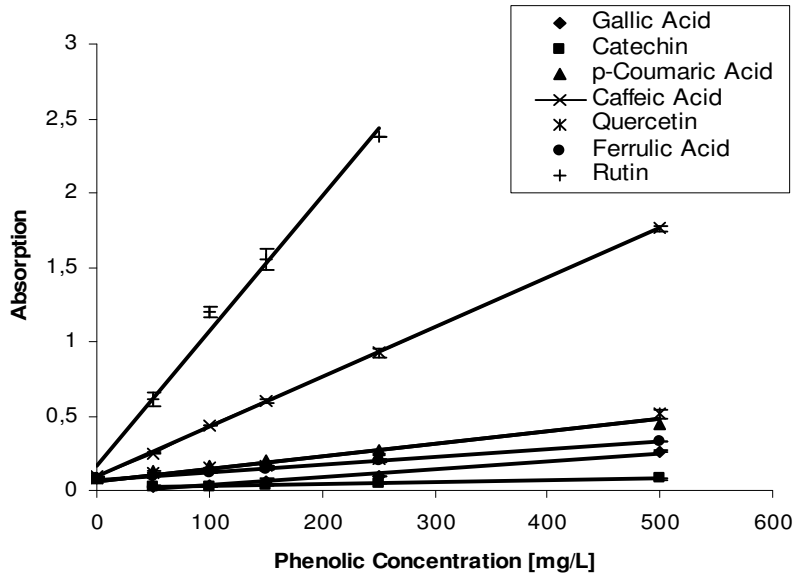


Figure 3. 22 Quinone absorption results of the CATPO reaction with phenolics for 10 sec reaction time (at 60°C, pH=7, E.Activity=40 U/L)

Table 3. 1 Analytical data referring to the calibration curve parameters of the CATPO, obtained when operating in the different solvent mixtures: (7 phenolics of wine)

Analyte	**Slope**	**Intercept**	r^2	**Linear Range(mg/L)**
Gallic Acid	0.0005	-0,0142	0,9780	50.0-500.0
Catechin	0.0001	0,0171	0,9942	50.0-500.0
Caffeic Acid	0.0033	0,0931	0,9999	0.0-500.0
p-Coumaric Acid	0.0007	0,0891	0,9994	0.0-500.0
Ferrulic Acid	0.0005	0,0740	0,9941	0.0-500.0
Quercetin	0.0008	0,0606	0,9446	0.0-500.0
Rutin	0.0091	0,1645	0,9913	0.0-500.0

Calibration graphs were obtained for 7 phenolic compounds (gallic acid, catechin, caffeic acid, p-coumaric acid, quercetin, ferrulic acid, rutin) which gave response against CATPO of *S.thermophilum*. The calibration curves of phenolics are displayed in Figure 3.22 above as a representative of 7 phenolics, which were proved to be responsive. The main analytical data of calibration curve parameters of all phenolics are reported in Table 3.10.

Although gallic acid and quercetin showed lower linear correlations in comparison with previous results of reaction with CLTAs, relatively good linear correlations were obtained for all 7 phenolics of wine. Better linear ranges were achieved for caffeic acid, *p*-coumaric acid, quercetin and rutin than the ones were obtained from reaction with CLTAs. Calibration curve slopes of caffeic acid and rutin are higher than other phenolics', as observed with CLTAs. This means that, reaction rates of these phenolics are higher than other phenolics'.

3.14 Effect of H_2O_2 on CATPO Activity

It was reported that, the enzyme obtained from *S.thermophilum* is a bifunctional catalase, with an additional catechol oxidase activity (Sutay *et al*., 2008) Thus, *S.thermophilum* enzyme exhibits a unique function, oxygen atom transfer from the peroxo intermediate of enzyme to phenol substrates, which is an attractive process of the enzyme. During enzyme catalyzes the dehydrogenation of catechols to the corresponding *o*-quinones (catecholase activity), O_2 must exist in the environment. Enzyme can convert hydrogen peroxide found in environment into oxygen. So the presence of H_2O_2 in the environment can accelerate the reaction of catechol oxidation and therefore the amount of product (o-quinone) could be redounded.

In order to see the effect of H_2O_2 on oxidation of phenols, two samples of the same concentration of gallic acid solution was prepared. H_2O_2 was added to first sample,

whereas buffer was put in the other solution instead. The enzyme of *S.thermophilum* was included in both of them. After 15 min, sulfuric acid was added to stop enzymatic reaction. It inactivates the active sites of enzyme. After that, final total phenolic contents of the solutions were measured by Folin-Ciocalteau method. The results obtained for two samples are reported in Table 3.11.

Table 3. 6 Total gallic acid concentration of samples before and after the reaction

	SAMPLE 1 (Gallic Acid Solution +H_2O_2+CATPO)	**SAMPLE 2** (Gallic Acid Solution +CATPO)
Initial Concentration (mg/L)	500	500
Final Concentration (mg/L)	276.5 ± 47.7	427.9 ± 12.1

Phenol oxidase activity of CATPO is very low in comparison with tyrosinase. So even if 15 min reaction is allowed, only 14.42 % saturation value had reached. However, when H_2O_2 was introduced in the environment, the saturation value raised to 44.7% which is 3 times of saturation value of CATPO. This can be explained as a result of increasing concentration of oxygen in solution.

CHAPTER 4

CONCLUSIONS & RECOMMENDATIONS

The aim of this study was to investigate the use of cross-linked enzyme (tyrosinase) aggregates (CLTA) obtained from crude mushroom extract for a rapid phenolic content analysis in wines and to compare phenolic characteristics of Turkish red wines. These CLTAs prepared from crude mushroom tyrosinase are targeted to be incorporated into a biosensor to detect polyphenol contents in aqueous environments. For this purpose, CLTAs were tested in the detection of phenolic compounds.

Ten commercially available red wines and three white wines produced from grapes of different regions of Turkey were analyzed in terms of their total phenol contents by both Folin-Ciocalteau assay and CLTAs. The results of the experiments were consistent with the real values obtained by Folin-Ciocalteau assay. Wines produced from Boğazkere and Karalahna grapes were found to contain highest amount of phenolic compounds among all, while Kalecik Karası led to the lowest phenolic content. Among white wines, on the other hand, the one produced from Emir grapes had the highest phenolic content.

Furthermore, CLTAs prepared from crude mushroom tyrosinase exhibited very high operational stability and retained more than 90% of its activity after 30th use. Besides, it showed good shelf-life stability for about 2 months by retaining 90% of its activity.

In addition to CLTAs, the enzyme (CATPO) obtained from a fungal microorganism, *S.Thermophilum*, was studied in this research for measurement of wine phenolics. It showed selectivity towards syringic and vanillic acid, as in the case of CLTAs.

Moreover, catalase-phenol oxidase activity (H_2O_2-dependent oxygenation of substrates) of the enzyme increased with the addition of H_2O_2 in the aqueous environment.

The presented bioanalytical measurement technique provides a rapid and reliable determination of phenolics in aqueous solutions such as wine. It was also shown that the CLTA is a stable and recyclable biocatalyst. Furthermore, automation of the presented method is easily achievable and will enable the control of total phenolics content during all process steps.

The CLTAs show promising results, and in future the method may be successfully incorporated into a biosensor to detect polyphenol contents in various aqueous environments. For further studies, more efforts on measurement of wine phenolics by using CATPO produced from *S.thermophilum* should be spent. Then, the studies to find a suitable enzyme to oxidize phenols that are not oxidized by fungal enzymes (CATPO & CLTA) are necessary. Co-immobilization of different enzymes is also possible.

REFERENCES

Amotz, S. (1987). Method for production of an immobilized enzyme preparation by means of a crosslinking agent. (Novo Industri A/S) US 4,665,028.

Arifoglu, N., Ogel, Z.B. (2000). Avicel-adsorbable endoglucanase production by the thermophilic fungus *Scytalidium thermophilum* type culture *Torula thermophila*. *Enzyme and Microbial Technology*, 27:560–569.

Aytar, B.S., Bakır, U. (2008). Preparation of cross-linked tyrosinase aggregates. *Process Biochemistry*, 43(2), 125-131.

Basly, J.P., Marre-Fournier F., Le Bail, J.C., Habrioux G., Chulia A.J. (2000). Estrogenic/antiestrogenic and scavenging properties of (E)- and (Z)-resveratrol. *Life Science*, 66, 769-777.

Bertrand, G. (1985). Sur la laccase et sur le pouvoir oxydant de cette diastase. *Comptes rendus de l'Académie des sciences (Paris)*, 120, 266–269.

Birnhaum, S. (1988). *Immobilized Macromolecules: Application potentials*. London: Springer Verlag.

Bloomfıeld, D.G., Heatherbell D.A., Pour N.M.S. (2003). Effect of *p*-coumaric acid on the color in red wine. *Mitteilungen Klosterneuburg Rebe und Wein, Obstbau und Früchteverwertung*, 53, 195-198.

Bollag, J.M., Liu, S.Y., Minard, R.D. (1982). Enzymatic oligomerization of vanillic acid. *Soil Biology and Biochemistry*, 14(2), 157-163.

Bonakdar, M., Vilchez, J.L., Mottola, H.A. (1989). Bioamperometric sensors for phenol based on carbon paste electrodes. *Journal of Electroanalytical Chemistry*, 266, 47–55.

Burke, R.M., Cairney, J.W.G. (2002). Laccases and other polyphenol oxidases in ecto- and ericoid mycorrhizal fungi. *Mycorrhiza*, 12, 105-116.

Cabirol, F.L., Hanefeld, U., Sheldon, R.A. (2006). Immobilized hydroxynitrile lyases for enantioselective synthesis of cyanohydrins: sol-gels and cross-linked enzyme aggregates. *Advanced Synthesis & Catalysis*, 348(12+13), 1645-1654.

Cabral, J.M.S., Kennedy, J.F. (1993). Immobilisation techniques for altering thermal stability of enzymes. In: *Thermostability of Enzymes*. M.N. Gupta Ed: 163-179, Berlin: Springer Verlag,.

Calera, J.A., Sanchez-Weatherby, J., Lopez-Medarano, R., Leal, F. (2000). Distinctive properties of the catalase B of *Aspergillus nidulans*. *FEBS Letters*, 475, 117-120.

Campanella, L., Sammartino, M.P., Tomassetti, M. (1992). New enzyme sensor for phenol determination in non-aqueous and aqueous medium. *Sensors and Actuators B- Chemical*, 7, 383–388.

Cao, L., van Langen, L.M., Janssen, M.H.A., Sheldon, R.A. (1999). Preparation and properties of cross-linked aggregates of penicillin acylase and other enzymes. *European Patent Applications*; EP1088887A1.

Cao, L., van Langen, L.M., Sheldon, R.A. (2003). Immobilised enzymes: carrier-bound or carrier-free?. *Current Opinion in Biotechnology*, 14, 387–394.

Cao, L., van Langen, L.M., van Rantwijk, F., Sheldon, R.A. (2001). Cross-linked aggregates of penicillin acylase: robust catalysts for the synthesis of b-lactam antibiotics. *Journal of Molecular Catalysis B: Enzymatic*, 11, 665-670.

Cao, L., van Rantwijk, F., Sheldon, R.A. (2000). Cross-linked enzyme aggregates: a simple and effective method for the immobilization of penicillin acylase. *Organic Letters*, 2, 1361-1364.

Chaplin, M.F., Bucke, C. (1990). Enzyme Technology. Great Britain: Cambridge University press.

Chevalier, T., de Rigal, D., Mbeguie-A-Mbeguie, D., Gauillard, F., Richard-Forget, F., Fils-Lycaon, B.R. (1999). Molecular cloning and characterisation of apricot fruit polyphenol oxidase. *Plant Physiology*,119, 1261–1269

Cheynier, V., Rigaud, J. (1986). HPLC separation and characterization of flavonols in the skins of *Vitis vinfera* var. Cinsault. *American Journal of Enology and Viticulture*, 37(4), 248–252.

Chmura, A., van der Kraan, G.M., Kielar, F., van Langen, L.M., van Rantwijk, F., Sheldon, R.A. (2006). Cross-linked aggregates of the hydroxynitrile lyase from Manihot esculenta: highly active and robust biocatalysts. *Advanced Synthesis & Catalysis*, 348(12+13), 1655-1661.

Clark, D.S. (1994). Can immobilisation be exploited to modify enzyme activity?. *Trends in Biotechnology*, 12, 439-443.

De Gaulejac, N.S., Glories Y., Vivas N. (1999). Free radical scavenging effect of anthocyanins in red wines. *Food Research International.,* 32, 327–333.

Donovan, J.L., Manach, C., Rios, L., Morand, C., Scalbert A., Rémésy C. (2002). Procyanidins are not bioavailable in rats fed a single meal containing grape seed extract or the procyanidin dimer B3. *British Journal of Nutrition*, 87, 299–306.

Durán, N., Esposito, E. (1997). Lignin biodegradation and effluent treatment by linginolytic fungi. In: *Microbiologia Ambiental*, I.S. de Melo, J.L. De Acevedo, Ed.: Cap. 12, 269-292, CNPMA EMBRAPA Editora.

Duran, N., Rosa, M.A., D'Annibale, A., Gianfreda, L. (2002). Applications of lactases and tyrosinases (phenoloxidases) immobilized on different supports: a review. *Enzyme and Microbial Technology*, 6179, 1–25.

Fenoll, L.G., Rodriguez-Lopez, J.N., Garcia-Sevilla, F., Garcia-Ruiz, P.A., Varon, R., Garcia-Canovas, F., Tudela, J. (2001), *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1548(1), 1-22.

Ferguson L.R, Zhu S., Harris P.J. (2005). Antioxidant and antigenotoxic effects of plant cell wall hydroxycinnamic acids in cultured HT-29 cells. *Molecular Nutrition & Food Research*, 49(6), 585-93.

Frankel, E.N., Kanner, J., German, B., Parks, E., Kinsella, J.E. (1993). Inhibition of oxidation of human low-density-lipoprotein by phenolic substances in red wine. *The Lancet*, 341, 454-457.

Garcia-Falcon, M.S., Pérez-Lamela, C., Martinez-Carballo, E., Simal-Gandara, J. (2007). Determination of phenolic compounds in wines: Influence of bottle storage gof young red wines on their evolution. *Food Chemistry*, 105, 248-259.

Gaur, R., Pant, H., Jain, R., Khare, S.K. (2006). Galacto-oligosaccharide synthesis by immobilized Aspergillus oryzae b -galactosidase. *Food Chemistry*, 97(3), 426-430.

Goldberg, I., Hochman, A., (1989). Purification and characterization of a novel type of catalase from the bacterium *Klebsiella pneumoniae*. *Biochimica et Biophysica Acta*, 991, 330-336.

Gomez-Alonso, S., Garcia-Romero, E., Hermosin-Gutiérrez, I. (2007). HPLC analysis of diverse grape and wine phenolics using direct injection and multidetection by DAD and fluorescence. *Journal of Food Composition and Analysis*, 20, 618-626.

Halaouli, S., Asther, M.,.Sigoillot, J.C., Hamdi, M., and Lomascolo A. (2006). Fungal tyrosinases: new prospects in molecular characteristics, bioengineering and biotechnological applications. *Journal of Applied Microbiology*, 100, 219–232

Hall, G.F., Best, D.J., Turner, A.P.F. (1988). Amperometric enzyme electrode for the determination of phenols in chloroform. *Enzyme and Microbial Technology*, 10, 543–546.

Harkensee, D., Beutel, S., Young, M., Ulber, R. (2006). Development of a fast spectroscopic enzyme assay for on-site measurement of total polyphenol content in grapes and wine. *Analytical and Bioanalytical Chemistry*, 384, 1013-1018.

Hobbs, H.R., Kondor, B., Stephenson, P., Sheldon, R.A., Thomas, N.R., Poliakoff, M. (2006). Continuous kinetic resolution catalysed by cross-linked enzyme aggregates, 'CLEAs ', in supercritical CO_2. *Green Chemistry*, 8(9), 816-821.

Illanes, A., Wilson, L., Altamirano, C., Cabrera, Z., Alvarez, L., Aguirre, C. (2007). Production of cephalexin in organic medium at high substrate concentrations with CLEA of penicillin acylase and PGA-450. *Enzyme and Microbial Technology*, 40(2), 195-203.

Illanes, A., Wilson, L., Caballero, E., Fernandez-Lafuente, R., Guisan, J.M. (2006). Crosslinked penicillin acylase aggregates for synthesis of beta-lactam antibiotics in organic medium. *Applied Biochemistry and Biotechnology*, 133, 189-202.

Isobe, K., Inouse, N., Takamatsu, Y., Kamada, K., Wakao, N. (2006). Production of catalase by fungi growing at low pH and high temperature. *Journal of Bioscience and Bioengineering*, 101(1), 73-76.

Jolivet, J., Arpin, N., Wichers, H.J., Pellon, G. (1998) *Agaricus bisporus* browning: a review. *Mycological Research*, 102, 1459–1483.

Karakaya, S., El, S.N., Taş, A.A. (2001). Antioxidant activity of some foods containing phenolic compounds. *International Journal of Food Sciences and Nutrition*, 52, 501-508.

Kıralp, S., Cirpan, A., Toppare, L. (2005). Enzyme Electrodes for Determination of Total Phenolic Capacity of Red Wines. Journal of Applied Polymer Science, 98, 521-524.

Kıralp, S., Toppare, L., Yağcı, Y. (2003). Immobilization of Polyphenol Oxidase in Conducting Copolymers and Determination of Phenolic Compounds in Wines with Enzyme Electrodes. *International Journal of Bioogical Macromolecules,* 33, 37

Kıralp, S., Toppare, L., Yağcı, Y. (2004). Determination of Phenolic Compounds n Wines with Enzyme Electrodes Fabricated by Immobilization of Polyphenol Oxidase in Conducting Copolymers. *Designed Monomers & Polymers*, 7,3

Kikugawa, K., Hakamada, T., Hasunuma, M., Kurechi, T. (1983).Reaction of *p*-hydroxycinnamic acid derivatives with nitrite and its relevance to nitrosamine formation. *Journal of Agricultural and Food Chemistry*, 31(4), 780-5.

Kondo, K., Matsmoto, A., Kurata, H., Tanahashi, H., Koda, H., Amach, T., Itakura, H. (1994). Inhibition of oxidation of low-density-lipoprotein with red wine. *The Lancet (British edition)*, 344,1152 (1994).

Kong, P.J., Park, J.I., Kwon, O.Y., Han, Y.Y., Kim, S.Y., Lee, S.N., Jeong, H.S., Kim, S.S. (2007). Comparison of Inhibitory Potency of Various Antioxidants on the Activation of BV2 Microglial Cell Lines Induced by LPS. *Korean Journal of Physiology & Pharmacolog,* 11, 9.

Lee, J., Tarara, J.M. (2007). Grape and wine phenolics: a refresher. Washington Association of Wine Grape Growers Annual Meeting. http://www.ars.usda.gov/SP2UserFiles/person/37108/Jungmin_Lee_Presentationwawgg.pdf. Cited 20 June 2008.

Lee, K.M., Blaghen, M., Samama, J.P., Biellmann, J.F. (1986). Cross-linked crystalline horse liver alcohol dehydrogenase as a redox catalyst: activity and stability towards organic solvent. *Bioorganic Chemistry*, 14, 202-210.

Li, J., Chia, L.S., Goh, N.K., Tan, S.N. (1998). Silica sol-gel immobilized amperometric biosensor for the determination of phenolic compounds. *Analytica Chimica Acta*, *362* 203–211.

Liu, S.Y., Minard, R.D., Bollag J.M. (1981). Oligomerization Of Syringic Acid, A Lignin Derivative, By A Phenoloxidase. *Soil Science Society of America Journal*, 45(6), 1100-1105.

López, M., Martinez, F., Del Valle, C., Orte, C., Miró, M. (2001). Analysis of phenolic constituents of biological interest in red wine by high-performance liquid chromatography. *Journal of Chromatography A*, 922,, 359-363.

Lutz, M., Burestedt, E., Emneus, J., Liden, H., Gobhadi, S., Gorton, L., Marko-Varga, G. (1995). Effects of different additives on a tyrosinase based carbon-paste electrode. *Analytica Chimica Acta,* 305, 8–17.

Margolin, A.L. (1996). Novel crystalline catalysts. *Trends in Biotechnology*, 14, 223-230.

Mateo, C., Chmura, A., Rustler, S., van Rantwijk, F., Stolz, A., Sheldon, R.A. (2006). Synthesis of enantiomerically pure (S)-mandelic acid using an oxynitrilase-nitrilase bienzymatic cascade: a nitrilase surprisingly shows nitrile hydratase activity. *Tetrahedron: Asymmetry*, 17(3), 320-323.

Mazocco, F., Pifferi, P.G. (1976). An improvement of the spectrophotometric method for the determination of tyrosinase catecholase Activity by besthorn's hydrazone. *Analytical Biochemistry*, 72, 643-647.

Messing, R.A. (1975). *Immobilized Enzymes for Industrial Reactors*. London: Academic Press.

Montavon, P., Kukic, K.R., Bortlik, K. (2007). A simple method to measure effective catalase activities: Optimization, validation, and application in green coffee. *Analytical Biochemistry*, 306, 207-215.

Montereali, M.R., Vastarella, W., Seta, L.D., Pilloton, R. (2005). Tyrosinase biosensor based on modified screen printed electrodes: measurements of total phenol content. *International Journal of Environmental Analytical Chemistry*, 85, 795-806.

Nakamura, T., Sho, S. and Ogura, Y. (1966). On the purification and properties of mushroom tyrosinase. *The Journal of Biochemistry*, 59, 481–486.

Ogel, Z.B., Yüzügüllü, Y., Mete, S., Bakır., U., Kaptan, Y., Sutay, D., Demir, A.S. (2006). Production, properties and application in biocatalysis of a novel extracellular alkaline phenol oxidase from the thermophilic fungus *Scytalidium thermophilum*. *Applied Microbiology and Biotechnology*, 71(6), 853-862.

Ortega, F., Dominguez, E., Önsson-Pettersson, G., Gorton, L. (1993). Amperometric biosensor for the determination of phenolic compounds using a tyrosinase graphite electrode in a flow injection system. *Journal of Biotechnology*, 31, 289–300.

Önnerfjord, P., Emnéus, J., Marko-Varga, G., Gorton, L. (1995). Tyrosinase graphite-epoxy based composite electrodes for detection of phenols. *Biosensors and Bioelectronics*, 10, 607–619.

Packer, L. (1984). *Methods in Enzymology Vol 105*, 1st Ed., Orlando, USA: Academic press Inc.

Price, S.F., Breen, P.J., Valladao, M., Watson B.T. (1995). Cluster sun exposure and quercetin in Pinot noir grapes and wine. *American Journal of Enology and Viticulture*, 46**,** 187–194.

Quinn, K.M., Singleton, V.L. (1985). Isolation and identification of ellagitannins from white oak and an estimation of their roles in wine. *American Journal of Enology and Viticulture*. 36, 148–155.

Quiocho, F.A., Richards, F.M. (1964).Intermolecular cross-linking of a protein in the crystalline state: carboxypeptidase A. *The Proceedings of the National Academy of Sciences (USA)*, 52, 833-839.

Quiocho, F.A., Richards, F.M.(1966). The enzyme behaviors of carboxypeptidase-A in the solid state. *Biochemistry*, 5, 4062-4076.

Rajendhran, J., Gunasekaran, P. (2007). Application of cross-linked enzyme aggregates of Bacillus badius penicillin G acylase for the production of 6-aminopenicillanic acid. *Letters in Applied Microbiology*, 44(1), 43-49.

Ray Sahelian, M.D. (2007). Health benefit of Rutin supplement. *Fundamental & Clinical Pharmacology*, 21(5), 521-9.

Rescigno, A., Sanjust, E., Montanari, L., Sollai, F., Soddu, G., Rinaldi, A.C., Oliva, S., Rinaldi, A. (1997). Detection of laccase, peroxidase and polyphenol oxidase on a single polyacrylamide gel electrophoresis. *Analytical Letters*, 30, 2211-2220.

Ritchey, J.G., Waterhouse, A.L. (1999). A standard red wine: monomeric phenolic analysis of commercial Cabernet Sauvignon wines. *American Journal of Enology and Viticulture,* 50, 91–100.

Robb, D.A., Gutteridge, S. (1981). The polypeptide composition of two fungal tyrosinases. *Phytochemistry*, 20, 1481–1485.

Roberge, C., Fleitz, F., Pollard, D., Devine, P. (2007). Asymmetric synthesis of cyanohydrin derived from pyridine aldehyde with cross-linked aggregates of hydroxynitrile lyases. *Tetrahedron Letters*, 48(8), 1473-1477.

Robichaud, J.L., Noble, A.C. (1990). Astringency and bitterness of selected phenolics in wine. *Journal of the Science of Food and Agriculture*, 53, 343–353.

Rocchietti, S., Urrutia, A.S.V., Pregnolato, M., Tagliani, A., Guisan, J.M., Fernandez-Lafuente, R., Terreni, M. (2002). Influence of the enzyme derivative preparation and substrate structure on the enantioselectivity of *penicillin G acylase*. *Enzyme and Microbial Technology*, 31, 88-93.

Rodriguez-Lopez, J.N., Escribano, J., Garcia-Canovas, F. (1994). A continuous spectrophotometric method for the determination of monophenolase activity of tyrosinase using 3-methyl-2-benzothiazolinone hydrazone. *Analytical Biochemistry*, 216, 205-212.

Romero-Perez, A.I. *et al*. (1996). Levels of *cis*- and *trans*-resveratrol and their glycosides in white and rose *Vitis vinifera* wines from Spain. *Journal of Agricultural and Food Chemistry*, 44, 2124–2128.

Rothstein, F. (1994). Differential precipitation of proteins: science and technology. In *Protein Purification Process Engineering*. R.G. Harrison Ed: 115-208 New York: Marcel Dekker, Inc.

Sanchez-Ferrer, A., Rodriguez, J.N., Garcia-Cànovas, F., Garcia-Carmona, F. (1995). Tyrosinase: a comprehensive review of its mechanism. *Biochimica et Biophysica Acta – Bioenergetics*, 1247, 1–11.

Schramm, D.D., Donovan, J.L., Kelly, P.A., Waterhouse, A.L., German, J.B. (1998). Differential effects of small and large molecular weight wine phytochemicals on endothelial cell eicosanoid release. *Journal of the Science of Food and Agriculture,* 46**,** 1900–1905.

Seo, S.Y., Sharma, V.K., Sharma, N. (2003). Mushroom tyrosinase: Recent prospects. *Journal of Agriculture and Food Chemistry*, 51, 2837–2853.

Sheldon, R.A., Sorgedrager, M.J., Janssen, M.H.A. (2007). Use of cross-linked enzyme aggregates (CLEAs) for performing biotransformations. *Chemistry Today*, 25, 62-67.

Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu Reagent. *Methods in Enzymology*, 299, 152-178.

Soler-Rivas, C., Jolivet, S., Arpin, N., Olivier, J.M., Wihers, H.J. (1999). Biochemical and physiological aspects ofbrown blotch disease of *Agaricus bisporus*. *FEMS Microbiology Reviews*, 23, 591–614.

Steffens, J.C., Harel, E., Hunt, M.D., Thipyapong, P. (1998). Polyphenol oxidase. *Colloques Institut National de la Recherche Agronomique (France)* 87, (Polyphenols 96), 223.

Sutay-Kocabaş, D., Bakır, U., Philips, S.E.V., McPherson, M.J., Ögel, Z.B. (2008). Purification, characterization, and identification of a novel bifunctional catalase-

phenol oxidase from *Scytalidium thermophilum*. *Applied Microbiology and Biotechnology*, 79(3), 407 – 415

Tarola, A.M., Milano, F., Giannetti, V. (2007). Simultaneous determination of phenolic compounds in red wines by HPLC-UV. *Analytical Letters*, 40(12), 2433-2445.

Tembe, S., Karve, M., Inamdar, S., Haram, S., Melo, J., D'Souza, S.F. (2006). Development of electrochemical biosensor based on tyrosinase immobilized in composite biopolymeric film. *Analytical Biochemistry*, 349*,* 72–77.

Toumela, M., Vikman, M., Hatakka, A., Itavaara, M., (2000). Biodegradation of lignin in a compost environment: a review. *Bioresource Technology*, 72(2), 169-183.

Tüchsen, E., Ottesen, M. (1977). Kinetic properties of subtilisin type Carlsberg in the crystalline state. *Carlsberg Research Community*, 42, 407-420.

Tyagi, R., Batra, R., Gupta, M.N. (1999). Amorphous enzyme aggregates. Stability toward heat and aqueous-organic cosolvent mixtures. *Enzyme and Microbial Technology*, 24, 348-354.

van Langen, L.M., Oosthoek, N.H.P., van Rantwijk, F., Sheldon, R.A. (2003). Penicillin acylase catalysed synthesis of ampicillin in hydrophilic organic solvents. *Advanced Synthesis & Catalysis* 345(6+7), 797-801.

van Langen, L.M., Selassa, R.P., van Rantwijk, F., Sheldon, R.A. (2005). Cross-linked aggregates of (R)-Oxynitrilase: A stable, recyclable biocatalyst for enantioselective hydrocyanation. *Organic Letters*, 7(2), 327-329.

Vetrano, A.M., Heck, D.E., Mariano, T.M., Mishin, V., Laskin, D.L., Laskin, J.D. (2005). Characterization of the oxidase activity in mammalian catalase. *The Journal of biological Chemistry*, 280(42), 35372-35381.

Wada, S., Ichikawa, H., Tatsumi, K. (1993). Removal of Phenols from Wastewater by Soluble and Immobilized Tyrosinase. *Biotechnology and Bioengineering*, 42, 854-858

Walsh, G. (2002). *Proteins Biochemistry and Biotechnology*. West Sussex: John Wiley and Sons, Ltd.

Wang, J., Lu, F., Lopez, D. (1994). Tyrosinase-based ruthenium dispersed carbon-paste biosensor for phenols. *Biosens*ors and *Bioelectronics*, 9, 9–15.

Waterhouse, A.L. (2001). Determination of Total Phenolics, In: *Current Protocols in Food Analytical Chemistry*, I1.1.1-I1.1.8, Wrolstad, R.E., Wiley.

Waterhouse, A.L. (2002). Wine Phenolics. *Annals of the New York Academy of Sciences*, 957, 21–36.

Waterhouse, A.L., Teissedre, P.L. (1997). Levels of phenolics in California varietal wine. In *Wine: Nutritional and Therapeutic Benefits*. T. Watkins, Ed.: 12–23. Washington, DC: American Chemical Society.

Wichers, H.J., Gerritsen, Y.A.M., Chapelon, C.G.J. (1996). Tyrosinase isoforms from the fruitbodies of *Agaricus bisporus*. *Phytochemistry*, 43, 333–337.

Wiegant, W.M. (1992). Growth characteristics of the thermophilic fungus *Scytalidium thermophilum* in relation to production of mushroom compost. *Applied and environmental Microbiology*, 58(4), 1301-1307.

Wilson, L., Illanes, A., Abian, O., Pessela, B.C.C., Fernandez-Lafuente, R., Guisan, J.M. (2004). Co-Aggregation of penicillin G acylase and polyionic polymers: An easy methodology to prepare enzyme biocatalysts stablein organic media. *Biomacromolecules*, 5, 852-857.

Xu, F., Shin, W., Brown, S.H., Wahleithner, J.A., Sundaram, U.M., and Solomon, E.I. (1996). A study of a series of recombinant fungal laccases and bilirubin oxidase

that exhibit significant differences in redox potential, substrate specificity, and stability. *Biochimica et Biophysica Acta*, 1292, 303–311.

Yamada, K., Akiba, Y., Shibuya, T., Kashiwada, A., Matsuda, K., Hirata, M. (2005). Water purification through bioconversion of phenol compounds by tyrosinase and chemical adsorption by chitosan beads. *Biotechnology Progress,* 21, 823–829.

Yaropolov, A.I., Kharybin, A.N., Emneus, J., Marko-Varga, G., Gorton L. (1995). Flow-injection analysis of phenols at a graphite electrode modified with co-immobilised laccase and tyrosinase. *Analytica Chimica Acta*, 308, 137-144.

Yıldız, H.B., Kıralp, S., Toppare, L., Yagci, Y. (2005). Immobilization of tyrosinase in poly(ethyleneoxide) electrodes and determination of phenolics in red wines. *Reactive & Functional Polymers*, 63, 155–161.

Yoshida, H. (1883). Chemistry of lacquer (*Urushi*) part 1. *Journal of Chemical Society*, 43, 472–486.

Yu, H.W., Chen, H., Wang, X., Yang, Y.Y., Ching, C.B. (2006). Cross-linked enzyme aggregates (CLEAs) with controlled particles: Application to *Candida rugosa* lipase. *Journal of Molecular Catalysis B: Enzymatic*, 43(1-4), 124-127.

APPENDIX A

MEDIUM COMPOSITIONS

YpSs Agar Medium

4.0 g/L Yeast extract
1.0 g/L K_2HPO_4
0.5 g/L $MgSO_4.7H_2O$
20.0 g/L Agar

Preculture Medium

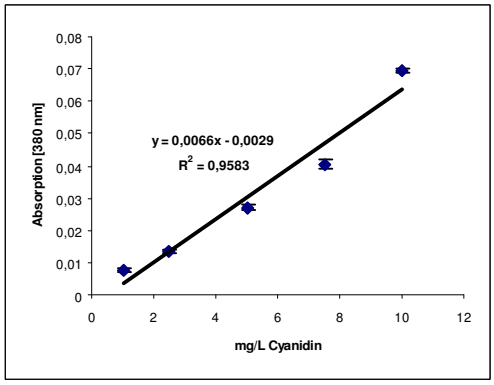
4.0 g/L Yeast Extract
1.0 g/L K_2HPO_4
0.5 g/L $MgSO_4.7H_2O$
10.0 g/L Glucose

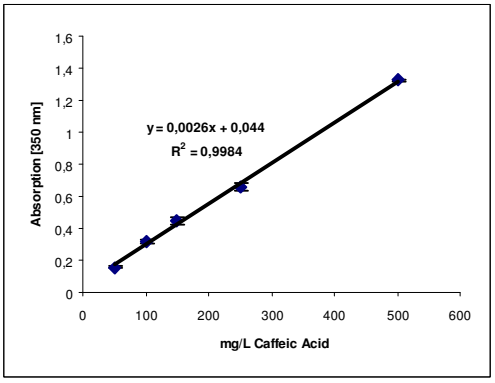
Mainculture Medium

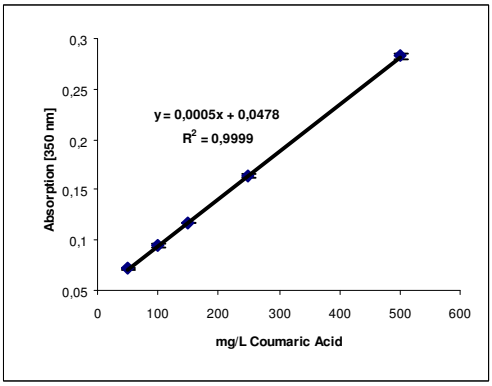
4.0 g/L Yeast Extract
1.0 g/L K_2HPO_4
0.5 g/L $MgSO_4.7H_2O$
0.1 g/L $CuSO_4.5H_2O$
10.0 g/L Glucose
(0.17 g/L Gallic Acid)

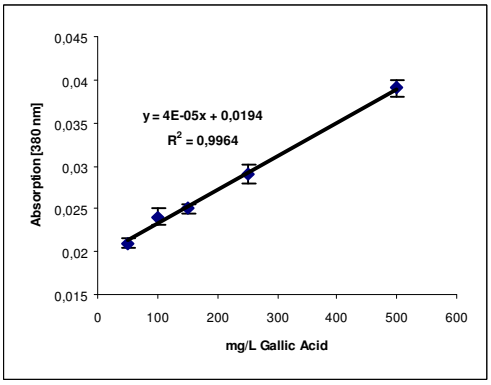
APPENDIX B

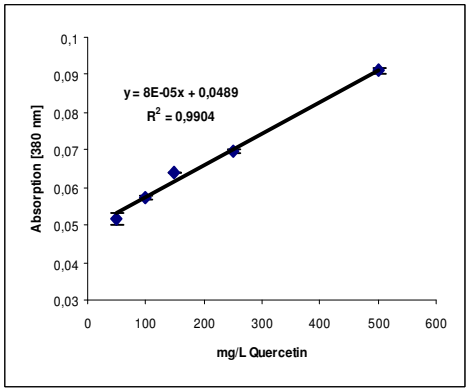
CALIBRATION CURVES FOR CLTA

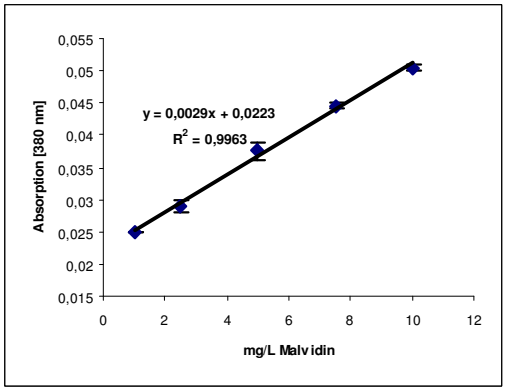


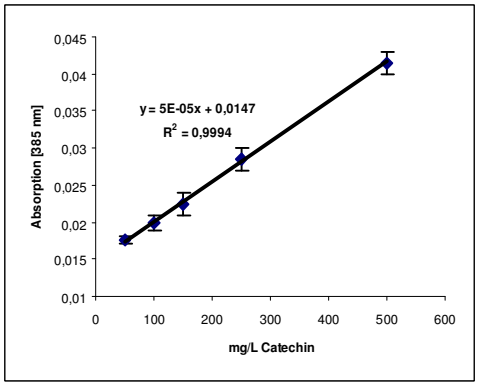


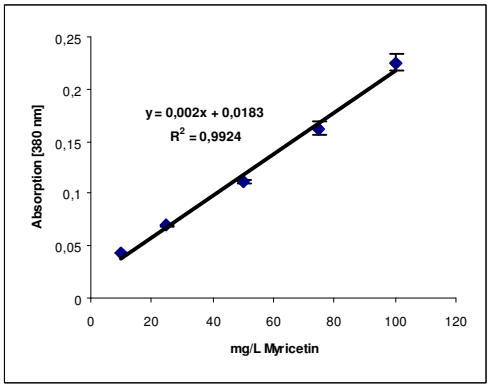





y = 8E-05x + 0,0489
R2 = 0,9904
Absorption [380 nm]
mg/L Quercetin


y = 0,0029x + 0,0223
R2 = 0,9963
Absorption [380 nm]
mg/L Malvidin

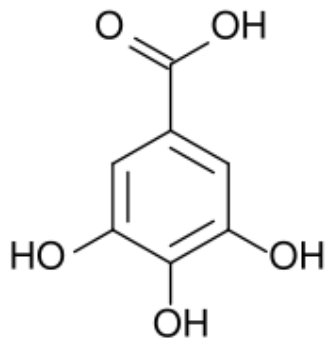

y = 5E-05x + 0,0147
R2 = 0,9994
Absorption [385 nm]
mg/L Catechin


y = 0,002x + 0,0183
R2 = 0,9924
Absorption [380 nm]
mg/L Myricetin

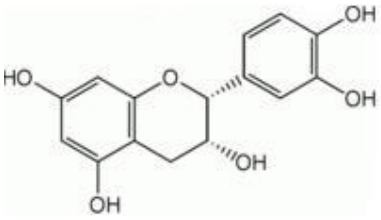
APPENDIX C

MOLECULAR STRUCTURES OF PHENOLICS

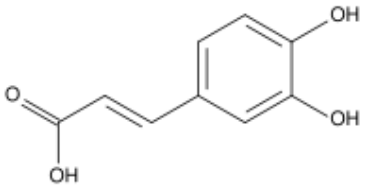
a.Gallic Acid



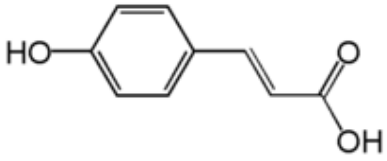
b.Catechin



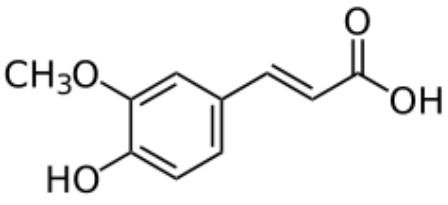
c.Caffeic Acid



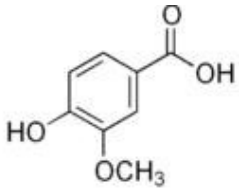
d. *p*-Coumaric Acid



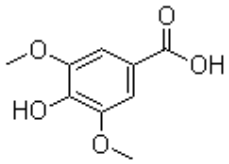
e. Ferulic Acid



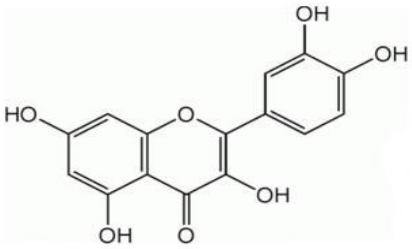
f.Vanillic Acid



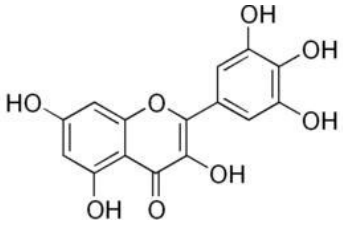
g. Syringic Acid



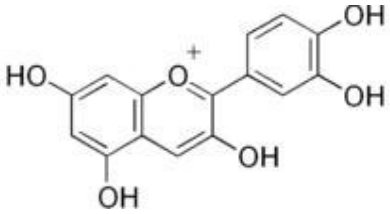
h.Quercetin



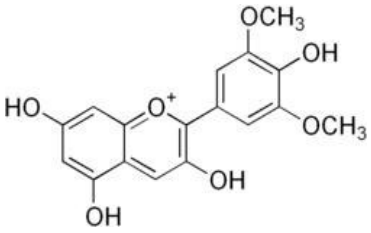
i. Myricetin



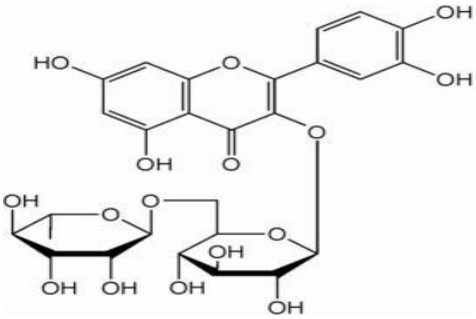
j. Cyanidin



k. Malvidin



l. Rutin

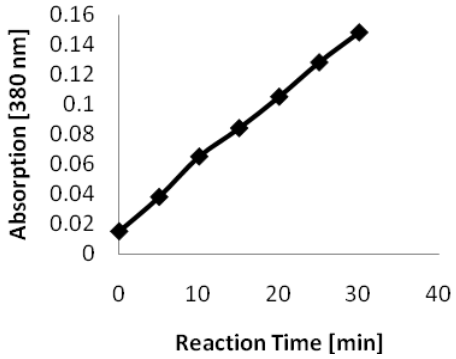


APPENDIX D

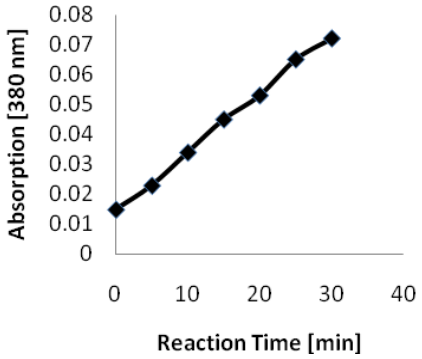
SATURATION CURVES

1. Crude Mushroom Tyrosinase (Catecholase Act=6.49 U/mL) Reaction

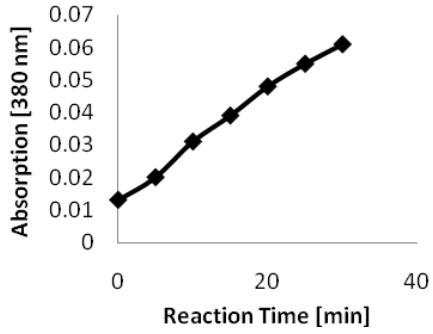
a)1 mM Gallic Acid Solution



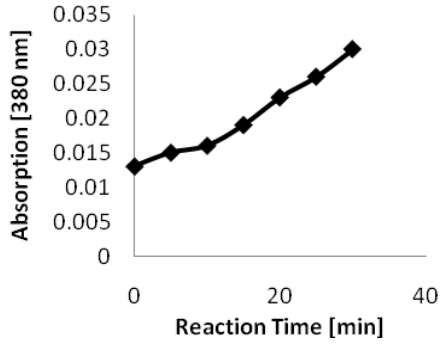
b) 0.5 mM Gallic Acid Solution



c) 0.25 mM Gallic Acid Solution

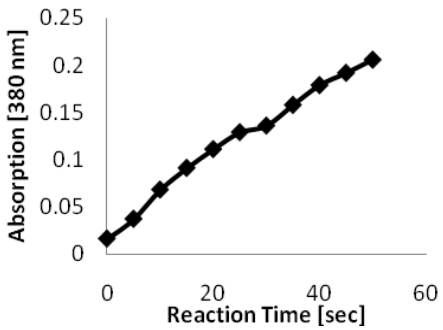


d) 0.125 mM Gallic Acid Solution

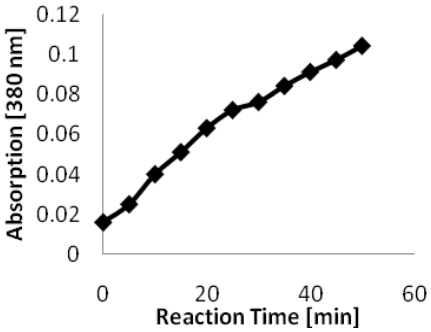


2. Crude Mushroom Tyrosinase (Catecholase Act=18.67 U/mL) Reaction

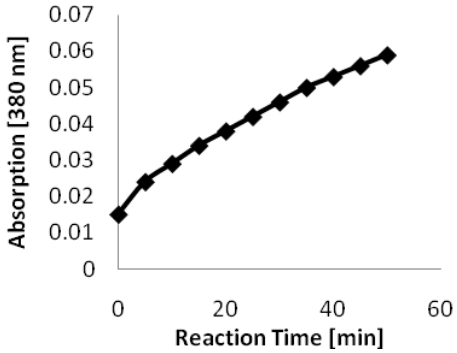
a) 0.5 mM Gallic Acid Solution



b)0.25 mmol Gallic Acid Solution

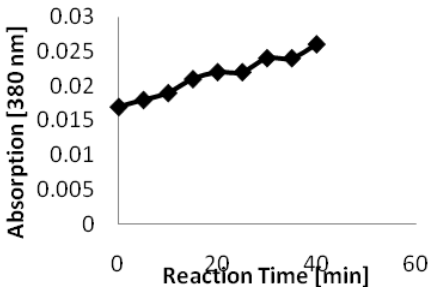


c) 0.125 mM Gallic Acid Solution



3. Crude Mushroom Tyrosinase (Catecholase Act=3.75 U/mL) Reaction

a) 0.04 mM Gallic Acid Solution



b) 0.03 mM Gallic Acid Solution